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Human guinea pigs have a choice

The British Medical Research Council is to carry out a five-year, controlled trial of vitamin supplements in the prevention of neural tube defects. But it should still adopt more open discussion of the trial.

Should people ever be used as guinea pigs in the investigation of a new drug or some new medical treatment? That is one of the questions on which opposition has been focused in the past few months to the proposed British trials of the efficacy of vitamin supplements, including folic acid, as a means of preventing neural tube defects (including spina bifida) in human births. The simple answer to what is an over-simple question is yes. People should be used as if they were experimental animals in proving new medical treatments. Moreover, people are already being used widely for just that purpose. But the analogy with guinea pigs is false. Guinea pigs are not able to choose whether to be part of some controlled trial in the laboratory. People invited to help test some novel treatment are, on the other hand, first given an explanation of what is intended and then an opportunity to decide whether or not they will participate. This is why it is right, if nevertheless courageous, that the Medical Research Council should have decided to go ahead with its long-planned trial of vitamin preparations as a means of preventing the recurrence of spina bifida births.

The planned trials are nevertheless unusual for several reasons. The incentive is the appearance in the past few years of evidence to suggest that supplements of vitamins, including folic acid during the first third of pregnancy, will help to prevent the recurrence of neural tube defects. The evidence (see *Nature* 2 December, p.396) is impressive but not conclusive. The planned trial is therefore based on the choice of four groups of women, each of whom has already given birth to a defective child, to which would be administered four different treatments — folic acid alone, folic acid plus more familiar vitamins, vitamins without folic acid and, for the remaining group, a placebo devoid of vitamins. The objective, of course, is not merely to discover whether one of these treatments will reduce the recurrence of neural tube defects but also to find some way of avoiding these defects among all women embarking on pregnancy. The controversy about the trial centres on the presence of the placebo group. Why, the critics ask, deprive one quarter of the women in the trial of any treatment when there is all this evidence that something in the original vitamin cocktail has some effect?

The assumption underlying this question is unfortunately just that. The control groups in the previous studies have not been control groups at all, but merely groups of women who have already had one defective child and who might have been offered vitamin supplements if they had not become ineligible, usually by already being pregnant (and only occasionally by refusal). That there may be some kind of self-selection in the assignment of women to the two groups is clear, although it is unlikely to have been significant. But certainly the women not given vitamin supplements in previous surveys, as in that now being carried out in the Republic of Ireland, are denied the benefits of the well-known Hawthorne effect, the improvement of virtually any social condition that comes about when something new is offered by experimentalists. Consequently, a trial without a placebo group would literally be incapable of yielding results bearing on the benefits that would obtain from the widespread administration of vitamin supplements to all women at risk of pregnancy, not merely those unfortunate enough to have produced an afflicted child. The point is especially important because there appears to be very little direct evidence of the untoward effects that there

may be of chronic administration of folic acid, and because it may in the end be necessary to administer whatever vitamin supplement is necessary by adding it to some general component of the diet, bread perhaps. Will those who now complain that a placebo group would be unethical in the forthcoming trial then take to the hustings to protest that it is unethical to add an untested material to everybody's diet?

So the case for going ahead with the Medical Research Council's trial is strong. The obvious alternative approach, the detailed investigation of the concentrations of various vitamins in different kinds of body cells at different times of life, but especially in pregnancy, would no doubt be valuable (and should not even now be neglected). The case for an independent investigation of the effects of the chronic administration of folic acid in laboratory animals is strong, but as a necessary complement to the trials now planned. But none of this could possibly yield, in the near future, guidance on what should wisely be done to reduce the incidence of neural tube defects in the population as a whole. Only the trial can do that.

But will the Medical Research Council be able to recruit the physicians and their patients whose cooperation will be necessary after all the controversy there has been? The council's statement last week says that it is anxious on this point. The burden of advising women whether to take part in the trials will after all fall on family physicians, not on the research council's officials. One particular difficulty will be that women who are unable to accept that a fetus diagnosed as carrying a neural tube defect could be aborted will be reluctant to take part. For others, the crucial consideration will be whether the proposed trials will be accompanied by thoroughly reliable means of diagnosing neural tube defects early in pregnancy. The Medical Research Council should not be afraid that the provision of such arrangements will expose it to the scurrilous charge that it is prepared to offer abortions to women taking part in the trial. In these circumstances, as in the ordinary ups and downs of pregnancy, such questions are questions between women and their physicians.

None of this implies that the Medical Research Council will have an easy passage in the five years ahead. While the council has been attacked unfairly on many occasions in the past few months, it should itself acknowledge that it has helped to exaggerate its own difficulties by its attempts to control open discussion of its plans. The pantomimic suppression of two letters from *Nature* correspondents a few weeks ago (see 25 November, p.310) was thoroughly unreasonable; it was probably, in the event, counter-productive. In the coming months, the council should resolve to be more open about its plans. For example, the protocols that have no doubt been drawn up to help decide when the trial should be abandoned, perhaps because significant evidence has accumulated in one direction or the other, should be openly published. Tactically, and so as not to compromise its other good works, the council should remind anyone who will listen that it is not the prime mover behind the spina bifida trials but rather the agent of the Department of Health, which originally set the ball rolling. But, for the long term, the council should also insist not merely on the need for this planned trial but also on the fact that advanced communities such as the British cannot look for all the benefits of modern medicine if they shy away from the need for trials in which some people are put at risk.



Ocean bed law disaccord

Several countries have not signed the Law of the Sea. A compromise must eventually be found.

The conference at Montego Bay in Jamaica to celebrate last week the signing of the Law of the Sea was by all accounts more like a wedding without the groom than a funeral without the corpse. The air of mutual congratulation was real enough. More than a hundred states turned up to sign the treaty; a third as many, including the United States and most of the industrialized states of Western Europe, stayed away. The issue that divides the two groups is now familiar: the arrangements in the treaty as negotiated for the exploitation of minerals on the sea-bed.

The disagreement is partly geographical. Within the newly defined "economic zone" of 200 miles around each maritime state, governments will be free to take what minerals they can find. Beyond that it will be necessary for those with the techniques for extracting minerals from the sea-bed to limit what they take but also to "share" their "technology" with other states that may share ambitions to win minerals from the deep oceans. While the provisions now put forward in the Law of the Sea are a good deal more realistic than those originally canvassed, they still give offence. The essential difficulty is that of making a bridge between the commercial interests of those who may in due course wish to retrieve minerals from depths of several kilometres, and who will presumably have to spend substantial amounts of their own money to reach a position in which they can do something worthwhile, and the objectives of those who are more concerned that the deep sea-bed should become a kind of international heritage of mankind. But too many of the schemes which are compromises between commercial interests and international idealism will work less than efficiently.

Several questions arise, the chief of which is that of who can be blamed for the way in which the Law of the Sea has not been signed by the potentially most important signatories. The impasse that has arisen was apparently well concealed at Montego Bay last week. The temptation now is to blame the United States for what has happened, and indeed the behaviour of the US delegation at the negotiations in New York earlier this year was a shameful mixture of indecisiveness and arrogance. But in reality what the Reagan Administration was saying that it would be a dangerous precedent if they were bound by the terms of an international treaty to share technology on terms unspecified and by means not understood and that the provisions of the Law of the Sea on deep-sea mining are merely a continuation by other means of the longstanding argument between the developing countries and those of the industrialized West. It would have been better for all concerned if these points had been made more openly much earlier in the negotiations.

So what happens next? Technically the treaty will come into force only when sixty of its signatories have taken the formal step of ratification, which is expected to take two years. Then, after a further five years, there will be a review conference at which the working of the treaty will be re-examined, and when it will be possible for those who have ratified the treaty to put forward amendments. Those not (repeat not) present at Montego Bay last week are now saying that they will nevertheless turn up at the review conference, if only as observers, to repair some of the damage that has been done. The snag is that without formal status as members of the treaty, they will be able to put forward new proposals only with the consent of the developing countries to which such offence has now been given.

Meanwhile, the signatories of the treaty are saying that as it now exists it is binding even on those who have chosen not to sign. The case for that position is weak. The signatories at Montego Bay last week may be right in saying that they could take non-signatory violators to the International Court of Justice at The Hague, but there is no reason to think they would win their cases.

So does the future hold merely a kind of licensed piracy on the sea-bed and even on the high seas? Given the likely pace of development of sea-bed mining, mineralogical piracy is unlikely.

The provisions on the rights of innocent passage in narrow straits are more immediately important. The danger, in the present stand-off between the signatories and the others, is that their disagreement may become open dispute at times of international tension. Would the rights of passage through the Red Sea of merchant ships from non-signatory states always be assured if there were some future conflict in the Persian Gulf? This is the kind of anxiety that should keep the non-signatory governments awake at night, and on the look-out for a more persuasive compromise than they have found so far.

Unorthodox assessments

A recently published book provides an intriguing glimpse of peer-review in action.

Like restaurant proprietors, journal editors have the right to refuse admission to anyone, the obligation to explain their attitude being merely moral. Dr Stefan Marinov has therefore performed a useful and courageous service by revealing in print* the manner in which journals (including this one) and funding organizations around the world respond to this obligation. For some years, Dr Marinov has submitted papers and proposals concerning "absolute space-time" to such bodies, with — by his own account — a rather small degree of success. Literal copies of this correspondence make up the bulk of his book and are absorbing reading.

It is an inevitable part of the duties of every editor to assess papers that lie near what may be called the fringe of science. Physics journals and "general" journals receive many attempts to "correct" the theory of relativity or to propose new laws underlying the structure and evolution of the Universe. Some papers are based on numerological mysticism or are in some other way so speculative and unpredictable as to justify immediate dismissal. Others contain mathematical errors. Some (as in Dr Marinov's case) contain detailed arguments, often including descriptions of experiments, which require more lengthy assessment.

Dr Marinov's book reveals some of his arguments and the reception they received. As a result, it is something of a handbook for editors faced with the need for an appropriate choice of words if the assessment process has revealed that rejection is the appropriate course of action. Does one follow, at one extreme, the rather curt approach of *Science* ("We decline to publish your paper on 'Measurement of the one-way velocity of light and the Earth's absolute velocity'. The manuscript is enclosed.") or, at the other, does one follow the editor of *Zeitschrift für Naturforschung* and (as Dr Marinov relates) exchange more than twenty letters and give the author an explanatory conversation over a pizza? Between these two lie the responses of some 35 other journals and the National Science Foundation (four referee reports followed by formal appeals up successive rungs of the hierarchy).

What can or should be learned from such a book? Those as tenacious and as radical in their scientific approach as Dr Marinov will learn what to expect in tone and quality from the variety of journals available to them. Those responsible for journals or funding bodies will gain a glimpse normally denied them of their colleagues in action. They may be drawn to conclude that to refuse publication without providing justification is to do less than justice to any author and plays into the hands of conspiracy theorists. They will note with disquiet that the more conscientious they are in this respect, the more conscientious a reply they will probably receive.

Publishers might conclude that there is a market for an unrefereed journal of unorthodox science. All readers should note how, in this case at least, the system seems to have ensured that orthodoxy prevails — deciding for themselves whether by malevolent conspiracy, inertia or good sense.

**The Thorny Way of Truth*, Est-Ovest International Publishers, Via Puggia 47/1, 16131 Genova, Italy.

Science to rescue EEC politics?

Davignon tackles the agricultural policy problem

Brussels

Scientific research in Europe seems poised to play a most unaccustomed role: as a tool in a major political issue, one which threatens the European Economic Community: the ballooning cost of the Common Agricultural Policy (CAP).

The CAP, designed in part to keep continental farmers on the land, consumes nearly three-quarters of the Community's budget, leads to enormous food surpluses, and threatens — politically — to blow the Community apart. Related policies of farming support in the United States also produce surpluses. Around 100,000 tonnes of butter, for example, are stored in a Kansas mountain; similar amounts cram cold stores in Europe. The consequent dumping of food on world markets is precipitating something approaching a transatlantic food trade war. Meanwhile, much of the world starves for want of rice, not butter or powdered milk, wine or maize.

The scientific initiative, designed to help solve the problems of CAP comes from the Vice President at the European Commission, Viscount Etienne Davignon — for whom everyone appears to jump when he wags a finger — and from his research director-general Professor Paolo Fasella. Between them they appear to be building the conceptual and political basis, both inside and outside the Commission, of a key role for research in agricultural policy, and, for that matter, in public health, which is another major financial headache for European governments.

These initiatives, still at an early stage (they are yet to be formally discussed by the 13 European commissioners), come after Davignon's success with Esprit, the Brussels programme for the information technologies. Davignon, assisted by Fasella's directorate, has steered Esprit through the labyrinth of EEC and national advisory committees, and around potential political opposition, with impressive speed. (The programme should start next year, only a year after conception. By comparison, an earlier and equally ambitious programme for biotechnology took seven years to reach this stage and is only just getting under way.)

The agriculture research proposals are to be based above all on a detailed analysis of the Community's agricultural budget — on what costs most and why. Beyond that, they relate — for the moment — to five central objectives.

The first objective is to reduce pro-

duction and distribution costs, so that farmers can make their profit and maintain their living standards on a smaller volume of produce; the second, to upgrade the product; the third, to seek alternative uses of land and people; the fourth, to take account of the specific food needs of developing countries — or, more prosaically, the existing and potential world food market. And the fifth objective is to develop non-food products, such as energy and materials, based on agricultural production.

For example, food storage losses are high, but might be reduced by a small amount of research and demonstration. On quality, Europe could aim to make a smaller quantity of a better wine, or to make high-value cheese from milk rather than low-value powder. ("It makes me mad that we distil wine and dry milk", says Fasella.)

On alternatives, farmers could grow new crops, aided by a little research on what would grow where and be profitable. Fasella himself, before he joined the Commission from biotechnological research in Italy, had a hand in a successful project north of Rome. There, farmers agreed to change from the production of price-supported wheat to strawberries and flowers, with the help of a little science and low technology.

And on agricultural industry, Europe could, for example, re-examine the use of wood as a material and chemical feedstock. "Lignin [a major wood-waste] is still too little studied", Fasella says.

Exactly when these and related proposals will bear fruit in the form of research contracts with the Commission is not clear, but the ideas are now roughly at the stage of

Esprit a year ago. Britain is pushing hard for reform of CAP. Most nations at least recognize its over-weight, and all might see the emerging research proposals as a useful aid to change — or to defusing a politically dangerous situation. If things moved as fast as Esprit, there could be tenders by 1984.

Besides agriculture, health is also on Commission minds. The cost of the European public health services (111,000 million European units of account a year — £61 million — for the 10 EEC countries except Greece, according to World Health Organization figures) could also be reduced by the right kind of research, Davignon and Fasella are arguing. The bulk of health service costs come in hospitalization, and only 14–15 per cent in drugs. The drug companies have had little motivation to develop preventive therapeutic measures — but the profitability of new drugs is declining rapidly. On both sides, therefore, and in other sectors of the health industry, there is an interest in a new approach. As in the agricultural sector, the Commission should start by an analysis of costs, Davignon and Fasella argue. From that point, financial priorities will determine research and development needs and ultimately new products.

Overly ambitious? "We are not aiming to be an eleventh state [beyond the ten of the EEC]", Fasella insisted last week. Rather, the Commission is determined to act as a strong service — to provide new, realistic means of action for member governments. And for the first time, it seems — if Davignon can convince his fellow commissioners — research and development are going to be serious tools of that action.

Robert Walgate

Carter appointees leave NSF

Washington

Edward A. Knapp, the new director of the National Science Foundation (NSF), has arranged the departure of the three other past presidential appointees at NSF. The surprise move apparently came at the request of the White House which aims to install its own appointees and replace "holdovers" from the Carter Administration.

Donald N. Langenberg, deputy director of the nation's basic science agency, which is traditionally viewed as nonpolitical, will leave by the end of the year, at the request of Knapp and the White House. Langenberg, who is on leave from the University of Pennsylvania, had been a candidate for the director's job. The sudden departure took even NSF's governing board by surprise.

A second Carter appointee, Eloise E. Clark, deputy director for biological, behavioural and social sciences, has

reportedly submitted a letter, stating her intention to resign, also at Knapp's urging.

Francis S. Johnson, deputy director for astronomical, atmospheric, earth and ocean sciences, also plans to go. His three-year leave of absence from the University of Texas at Dallas expires soon anyway.

The departures fuelled rumours that the Reagan Administration is politicizing the NSF. But a source close to the foundation said that "NSF is due for a surprisingly large increase in the fiscal 1984 budget, and Knapp was under pressure to have his own people there" in time for the announcement of the figure in January.

The chairman of the National Science Board, Dr Louis Bramscomb, said he had no information suggesting that the departures imply political influence but, he said, "that could show up if Ed Knapp is not given the independence to pick replacements with the highest professionalism."

Deborah Shapley

West German nuclear power

Fast breeder escapes veto

A vote in West Germany's federal parliament (*Bundestag*) last week promises to relieve pressures on the CDU/CSU/FDP coalition government by helping to remove the technical problems of atomic energy from political debate.

In last week's vote, the *Bundestag* finally agreed to lift the veto on the commissioning of the 800 MW fast breeder reactor at Kalkar. This veto was imposed in 1978 to prevent opponents in the SPD/FDP government from killing the project completely. A commission was appointed to inquire into the safety of the installation and the majority report, published last September, found that the risks of the sodium-cooled breeder at Kalkar were no greater than those of modern pressurized water reactors. The *Bundestag* thus had little choice but to permit start-up in 1983, although most of the SPD used the pretext of the minority opinion to vote against lifting the veto.

From its beginning in 1971, the entire project has been bedevilled by controversy. Delays over the siting of the reactor at Kalkar were followed by the parliamentary veto. The fast breeder project has also been a catalyst to the rise of the "Green" parties as a political force. Other, technical, problems have arisen over technology licensing and in proving the safety and containment in case of accident. According to the review carried out recently by management consultants A.T. Kearney and Motor Columbus for the Ministry of Research and Technology, mismanagement has also contributed to the increased costs and delays.

The overall result is that the costs for the project have been repeatedly revised upwards at a rate well above that of inflation, and now stand at DM 6,500 million (US \$2,700 million), up by DM 1,500 million since 1980. Earlier this year, it looked as if this reactor and the other, high temperature, prototype reactor were running out of money. But the government has now found enough to keep them going for 1983, partly through cutting back other energy projects. As a result, well over 10 per cent of the 1983 research budget will be spent on the two prototype reactors.

Further development will depend on investment by the utility companies, based on the *Länder*. They will judge the reactors on the financial returns and it appears at present that the fast breeder will not be competitive before the end of the century. Thus there is a suspicion that a different standard is being applied to nuclear energy from that for other energy research.

On the other hand, the high temperature reactor may interest a wider spectrum of industry, although the prototype has also been subject to delays and increases in cost.

The main interest in Germany is in the use of high temperature steam for the gasi-

fication of lignite and bituminous coal, of which there are extensive reserves. Combined heat and power schemes, already in use in some northern cities with conventional power stations, provide an outlet for waste heat. Finally the reactor might be used to drive thermochemical cycles for splitting water to produce hydrogen. The EEC Commission has made hydrogen a major research subject and several cycles are being studied at the Joint Research Centres and elsewhere.

J.S.Dunnett

European atom treaty

New rules

Brussels

France's agreement with India to supply the Tarapur nuclear power station is being questioned by officials in Brussels. The agreement may be breaking the Euratom Supply Agency's monopoly over the supply of nuclear materials which has existed since the Euratom treaty was signed in 1958. The inquiry could be used as a way of applying pressure on France to agree to a revision of Chapter 6 of the treaty which the European Commission is proposing in order to bring it in line with the changes that have taken place in the nuclear industry since 1958.

The new proposals are the third attempt in eight years to bring the treaty up to date. In the past both France and the United Kingdom have opposed such a move in

order to protect their nuclear fuel companies, British Nuclear Fuels Limited at Sellafield (Windscale) and Cogema at Cap de la Hague. The Commission suspects that both these companies have been using their market dominance to maximize profits by rigging prices and imposing unnecessary conditions on their customers in the rest of the European Community. Customers have been persuaded not to hold stocks of reprocessed fuels or to sell them to other users and the Commission feels that it would at once be realistic to abolish the illusion of a Euratom monopoly and to insist on a genuinely free market for the nuclear fuels industry. Otherwise there may be some risk that countries such as Belgium and West Germany may decide to build their own re-processing plants, which would lead to over-capacity in Europe.

The new rules which the Commission is putting forward would give the European Commission the power to penalize anyone imposing any restrictions on trade in nuclear materials inside the Community. Exports, on the other hand, could be contracted on a bilateral basis provided the Commission can ensure that the contract would not damage the interests of any other EEC country. But a certain solidarity amongst the EEC member states would continue to be enforced in the event of a shortage when the European Commission would undertake to share out stocks equitably. The Euratom Supply Agency, which in the past has been successful in negotiating long term uranium supply contracts with Australia, Canada and the United States, would continue to supervise and negotiate such deals. **Jasper Becker**

Stanford cuts comment on patent

Washington

Stanford University, citing "erroneous public impressions" concerning the Cohen-Boyer biotechnology patent, last week ordered the US Patent and Trademark Office to keep secret all future action on its pending application. The move came just before the patent office was expected to announce its latest action on the application, which it tentatively rejected last summer.

Normally, all actions on a patent application are secret until a patent is granted but Stanford originally waived its right to secrecy. According to Robert Rosenzweig, Stanford's vice-president for public affairs, the "central issue" in Stanford's decision to close the file was "the dispute over inventorship" (see *Nature* 25 November, p. 303). "We thought that had some potential for damaging people's reputations unnecessarily", he said. Dr Robert Helling, a co-author of the key scientific paper on which the patents are based, is now claiming that he is a co-inventor; the confusion over Helling's role was cited in the patent

office's tentative rejection.

Rosenzweig said that keeping the file open had been "an experiment with some risk" and that "the experiment has failed". He said, "when the application process is completed, the file will be opened for public inspection. Until then, it is better that it not emerge in piecemeal fashion."

Stanford has already sold licences to the first Cohen-Boyer patent, issued in December 1980, to 73 companies for an initial fee of \$10,000 per year. That patent, which covers the basic process for inserting foreign DNA into bacteria, is similar to the second one, now pending, which covers the transformed bacteria products. Thus there has been concern that the flaws the patent office cited in the second patent could invalidate the first patent. The licensees have discussed the possibility that Stanford should open the first patent for reexamination and meanwhile hold the licence fees in escrow. Rosenzweig said that the licensees would be kept informed of the progress of the second patent. **Stephen Budiansky**

Chemical warfare

Fight amongst Finnish

The publication last week of the report of the United Nations on allegations that the Soviet Union and Vietnam have been using chemical warfare against insurgents in Afghanistan and South East Asia has caused some embarrassment to the Finnish foreign minister Paer Stenbaeck, who refused to let his ministry assist the enquiry. According to the independent newspaper *Laensi-Savo*, the minister was "mixing up the work of Finland's Technical Research Institute with our foreign policy's credibility".

The implication from comments in the Finnish media is that there has been a conflict over the freedom to research, with Mr Stenbaeck forbidding the project and the head of the Technical Research Institute (VTT) Dr Pekko Jauho insisting on the research going ahead.

During the past ten years, Finland has built up, under the supervision of the foreign ministry, a chemical warfare-monitoring capability which involves a number of laboratories and institutions — mainly civilian — including VTT. This capability, the Finns hope, will ultimately be used under the auspices of international agreements banning chemical warfare. Meanwhile the Finns have built up a considerable body of experience, and it was

reasonable, after the pledges of successive Finnish governments to support the United Nations in every aspect of its peacekeeping role, that the UN enquiry team should approach the Finns for assistance in analysing vegetation and blood samples from South-East Asia.

But Mr Stenbaeck was reluctant to let his ministry help. He felt, apparently, that without the guaranteed conditions for impartial and neutral verification which would presumably be embodied in a future treaty, it could damage his ministry's credibility to be involved in such a contentious issue as the allegations of chemical warfare in South East Asia. In particular, he had considerable doubts about the provenance of the samples, which had been brought to Finland without prior notification or agreement. The sample delivered to the Finnish Foreign Ministry was accordingly returned to the United Nations unopened, apparently on Mr Stenbaeck's direct instructions.

The other two samples were delivered to two scientists working for VTT, Professor Tor-Magnus Enari, an expert on *Fusarium*, and Professor Rolf Rosenbere, a physicist working in neutron-activation analysis. VTT, a government establishment responsible to the ministry of trade

and industry, operates a wide programme of contract research and development for both Finnish and foreign customers, and the UN commission was accepted as a purely commercial proposition. The work duly went ahead, giving, as it turned out, entirely negative results. **Vera Rich**

UK nuclear power

PWRs "safe"

A new report from the Advisory Committee on the Safety of Nuclear Installations (ACSNI) concludes that the Central Electricity Generating Board (CEGB) will be able to reach acceptable standards of safety for Britain's first pressurized water reactor (PWR), but draws attention to a number of areas in which it feels further work will be needed. In this respect it is substantially in agreement with the Nuclear Installations Inspectorate (NII) report earlier this year.

Some of the points raised by the document are sure to be used by objectors at the forthcoming public inquiry into the proposed PWR at Sizewell to embarrass CEGB, but the board seems confident that the questions raised in the ACSNI report have been answered since the end of 1981 (the latest date for ACSNI's evidence). This may explain some of the report's apparent ambiguities. In particular, CEGB's design proposals in its Preconstruction Safety Report have already been modified to include a double-containment system for the reactor pressure vessel, in accordance with current French and German practice.

ACSNI, set up to report to the Health and Safety Commission, attempts only to review the technical work of others and considers only selected aspects of PWR safety. The areas considered, by separate study groups, were the integrity of the pressure vessel, the plant/operator interface, and fuel processing. Most of the potential problem areas highlighted by ACSNI lie in the first two categories. Besides echoing NII's insistence on the highest achievable quality control of steel components in the reactor vessel itself, the report asks for equivalent standards to be applied to the whole of the coolant system. Standards for testing the integrity of welds are questioned, and more work is requested on degraded core phenomena. One of ACSNI's most significant criticisms of the CEGB Preconstruction Safety Report concerns the possibility of multiple breaks in the steam generator tubes, a consideration apparently excluded by CEGB on the grounds of unlikelihood. Since that time such an accident has in fact happened, and resulted in the release of some radioactivity.

On the subject of the plant/operator interface (one of the main causes of the accident in the PWR at Three Mile Island) the report makes recommendations about management and training schemes, and

Japan plans DNA database

Washington

Japan's entry into the field of molecular genetics may include a central data bank for DNA sequences. Storage of these complex sequences in computers and their statistical comparison have opened up new analytical possibilities in the field.

Some Japanese scientists attending a recent workshop in Aspen, Colorado, expressed an interest in starting such a database, and the scientists who run the new US database at the Los Alamos National Laboratory have welcomed their interest. The Japanese Ministry of Education may decide on 7 January 1983 to fund the project.

The database, likely to be located at the University of Tokyo, could be linked electronically to the Los Alamos database and to that run by the European Molecular Biology Laboratory (EMBL) at Heidelberg, West Germany.

A crucial question is which sequences it should collect. It would clearly be useful to collect the sequences published — sometimes in Japanese — in the Japanese scientific literature, which are generally unavailable to the groups at Los Alamos and Heidelberg. On the other hand, the number of sequences published in the Japanese journals is so far rather small, so that the database may be expanded to include those published abroad as well.



The Los Alamos database is supposed to contain all published sequences by July 1983, according to the terms of the contract that the US National Institutes of Health awarded to Bolt, Beranek and Newman for the Los Alamos-based project, known as GenBank. Walter Goad, the principal investigator at Los Alamos, said last week that sequences with an estimated 750,000 bases had been entered into his system, and that by the July 1983 deadline, it may include as many as 2 million bases. The GenBank database has just been announced "as available to the public for a nominal fee".

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says that a simulator designed to train operators specifically in the control of PWRs should be operational before the reactor is commissioned. The CEGB is again happy that it can satisfy this request. The difficulty is, as the report points out, that no-one knows exactly how the reactor will behave in extreme fault conditions, so a simulation of such events can only be approximate.

NII has welcomed the ACSNI report as a valuable input to its own examination of the CEGB case, but says that all the areas mentioned by ACSNI are currently receiving attention anyway. The inaccessibility of detailed current CEGB safety proposals is one major headache for objectors at the inquiry. **Tim Beardsley**

US energy research

Cuts demand

Washington

The Office of Management and Budget (OMB), still putting the final touches to the President's budget for fiscal year 1984, has proposed substantial cuts in research spending on fossil and solar energy and energy conservation. The OMB proposal, which is still subject to an appeal by the new Secretary of Energy, was leaked last week by congressional sources.

The proposed cuts are similar to those proffered by OMB for the past three years and which Congress has chosen to ignore. In addition to eliminating all demonstration projects and all federal government involvement in service programmes (for example grants to communities to provide insulation of residences), the cuts would hit the following research areas:

- Fossil energy. Research on combustion, coal liquefaction and gasification, and coal clean-up would be cut from the current level of approximately \$300 million (as

approved by the House and Senate appropriations committees for fiscal year 1983) to \$106 million, the same level that the President requested for 1983. Most of these funds go to research contracts with industry.

- Solar energy. Long a target of Reagan Administration budget-cutters, solar research would also be held to the level of the 1983 request, \$72 million. Congress is heading for an appropriation of \$180 million for 1983. The Department of Energy itself had asked for \$87 million for fiscal year 1984.

- Conservation and renewable energy. OMB wants a limit of \$36 million on this programme which has already been severely cut by the removal of all non-research components. Of the \$383 million appropriated by Congress for conservation in fiscal year 1982, \$156 million was for research; the Department of Energy (DoE) requested \$51 million for fiscal year 1984.

DoE is reported to be planning an appeal, at least for the fossil and conservation budgets. The final budget will be released in January. **Stephen Budiansky**

Soviet economy

Size costs dear

Last week, an article in *Pravda* by N. Kulagin, a hitherto obscure researcher at the N.A. Voznesenskii Financial-Economic Institute in Leningrad, evoked a flood of speculation in the West that, under its new leadership, the Soviet Union might reverse its traditional bias towards larger and more comprehensive industrial enterprises. Such an article is a standard method of launching a new policy trend, without going as far as to condemn the old.

In this case, under the title "It is hard to be universal", Kulagin compares the Soviet preference for "gigantic

multiprofile plants" with the development in the West of small specialized factories which cooperate with major industry by providing specialized components and services.

The large Soviet enterprises, each with its own staff of repairmen, instrument makers and transport-workers, is, Kulagin pointed out, wasteful of resources — in the Soviet machine building industry, for example, some 38 per cent of staff are engaged in such ancillary work, compared with only 11 per cent in the United States.

The type of centralized planning practised in the Soviet Union, as well as the vast investment originally needed to bring a number of sectors up to world level, undoubtedly has had much to do with the preference for gigantic plants. Such factories produce not only the goods specified in the State Plan, but also a large part of the components and auxiliary equipment they require. As Kulagin points out, these cause considerable difficulties but do not show up in the total output figures of the plant, thus making nonsense of the economic indicators.

Furthermore (a point not developed by Kulagin) if a "giant" industrial plant in Leningrad needs say, bolts with special performance parameters, it will commission some Leningrad institute to do the appropriate research, while a plant in Vladivostok, needing a precisely similar bolt, will commission the appropriate institute there. The duplication of research is considerable, particularly because, officially, Soviet scientists are not supposed to discuss their unpublished results with colleagues from other institutes. The introduction of small-scale plants, each specializing in the production of specific components, as advocated by Kulagin, could cut research costs considerably.

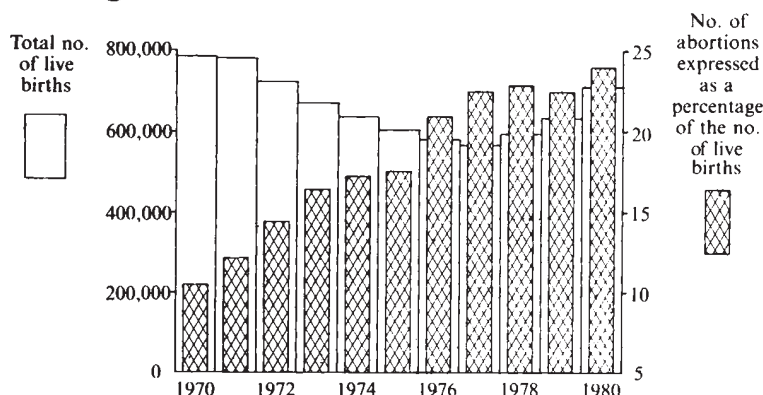
The implementation of the latest results of research and development — the major bottleneck in the modernization of Soviet industry — could also be carried through more effectively, in a network of small-scale plants, since the individual shut-downs for retooling could be better staggered.

And, perhaps most important, according to Kulagin, the costs of components made in one's "own" factory are frequently double what they would be from a "specialized" factory.

The concept of a changeover to smaller production units is not — in spite of the commentators last week — entirely new. The trend has slowly been taking shape over the past ten years, and several planning conferences have been held on the theme, one of which proposed the formation of a special Ministry of Interbranch Production to run the small component-producing plants. This has so far, however, failed to materialize, owing to bureaucratic difficulties inherent in the transfer of plants from one ministry to another.

Vera Rich

Increasing abortion rate in Britain



The annual number of abortions performed in England and Wales has risen from 82,730 in 1970 to 159,503 in 1980. In 1979 25 per cent of abortions were performed on women aged under 20 although only 9 per cent of all mothers fall inside this bracket. Not surprisingly, 60 per cent of the abortions were performed on women of between 20 and 34 — the age bracket which includes 85 per cent of mothers. The highest abortion rate is for women over 45 where the number of abortions actually exceeds the number of births. Source: *Health and Personal Social Services Statistics for England 1982* (HMSO: £7.50).

UK engineering

Institutional scrap continues

Britain's 200,000 chartered engineers are currently voting in a postal ballot on a recommendation from the board of the Council of Engineering Institutions (CEI) that control of its register of accredited engineers should be handed over from its own Engineers' Registration Board to the new Engineering Council, set up in the wake of the Finniston report. The outcome of the vote is far from certain; however, it is clear that if the recommendation is approved, CEI, which represents members of all the chartered engineering institutions, will have little reason to continue its existence.

The objects of the Engineering Council, listed in its Royal Charter, are to "advance education in and promote the science and practice of engineering . . . for the public benefit". Its members, who are appointed initially by the Secretary of State for Industry, will form a number of standing committees. The main purpose of these committees is to consult with industrial companies and educational establishments as well the existing professional institutions.

Engineering Council on 30 September detailed its future intentions in all its areas of responsibility including the setting up of a register of engineers. The statement clearly was influenced by the previously expressed concern of CEI that the views of its members should be assured of an adequate hearing on the new council and that existing titles would be protected. CEI will ask the existing professional institutions to oversee the awarding of titles, and their independence is explicitly recognized.

However, the Engineering Council's document fails to satisfy the expressed wishes of CEI that the titles of Technician Engineer and Engineering Technician should be awarded only to members of institutions. Furthermore, the document fails to guarantee the CEI's wish for self-regulation for the profession. Despite this, the CEI board approved the move with a 25 to 10 vote, although some members apparently felt they had very little choice. Now a two-thirds majority in the postal ballot of members is needed to implement the transfer.

Mr Gerald Mortimer, CEI chairman, is satisfied that "somehow or other" the views of professional engineers will gain an adequate hearing in the new Engineering Council. The move has been welcomed by the Fellowship of Engineering, which points, out that "it will be essential for professional engineers to use their influence in the institutions to maintain standards".

Tim Beardsley

US research

Basic physics in doldrums

Washington

Basic physics research in the United States is declining in quality compared with Europe, according to a new study by the US Office of Naval Research (ONR). Moreover, US industry is "just not playing the game" in basic physics, with the exceptions of Bell Laboratories and the IBM Corporation. Finally, the study suggests that the US Department of Defense has supported less and less of the important contributions to basic physics in the past decade.

These are the gloomy findings of a study counting the affiliations of all the contributors to *Physical Review Letters* (PRL), a leading journal of research in basic physics, between 1958 and 1979. Author Ted G. Berlincourt of ONR and his colleagues also counted the affiliations of contributors to the leading journal for applied physics, *Applied Physics Letters* (APL), from the time it was founded in 1962 until 1979 and found the same trend.

The accompanying figure shows the most striking finding of the PRL count. The journal published an increasing number of papers each year until 1968, when the number levelled off at approximately 1,000. Yet from the mid-1960s onward, the number of foreign contributors rose steadily and was still rising in 1979 when the count stopped. The number of US contributors to the journal decreased correspondingly.

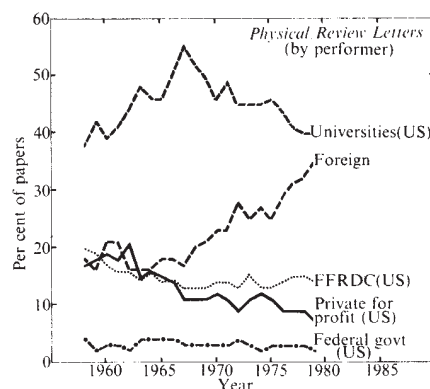
The ONR study speculates that this dramatic shift in foreign contributions might be caused by editorial policy. But George Trigg, who has been editor of *Physical Review Letters* since 1966, says no decision was made either to take more foreign contributions or to level off at 1,000 papers per year. "It has always puzzled me, because in the last 15 years the numbers of articles in other journals has increased" Trigg said. Apparently, no more than 1,000 are good enough to get through PRL's review system. The probable conclusion is that fewer and fewer US papers have passed muster.

Institutions outside the United States whose scientists are most successful in publishing in PRL are, in order, the Kernforschungsanlage Jülich in West Germany, the Atomic Energy Agency of Canada, and IBM Switzerland.

Berlincourt and his co-authors see several alarming trends emerging from their study. The "vigor" shown by basic physics outside the United States, and the parallel "vigor" of applied physics, "will without doubt be translated into stiff competition in all technology-dependent international arenas".

The applied physics work of US industry, they note, is "near stagnation". Private companies in the United States have contributed a declining proportion of

the papers in *Applied Physics Letters*. There has also been a decline in industry's contributions to *Physical Review Letters*; when Bell Laboratories' and IBM's contributions to PRL are taken out, industry has been contributing only 20 papers per year to PRL. IBM's and Bell Laboratories' contributions have declined since 1975.



From: "Performer affiliations and funding sources for research" by Ted G. Berlincourt, Harold Weinstock, Francis Narin, and Samuel R. Reisher.

The paper also notes that the number of physicists supported by the Department of Defense (DoD) contributing to PRL, the basic journal, declined precipitously after 1967, the year when the Mansfield Amendment determined that DoD should support only research directly relevant to military requirements.

Although the Mansfield Amendment was in effect for only two years, the trend it accelerated "had not been reversed by 1979, some eight years after the expiration of the Mansfield Amendment", even though senior defence officials since the Ford Administration have been saying they wish to rebuild DoD's ties to the university basic science community.

"The very marked erosion of DoD support for PRL-type basic physics . . . implies a significant decoupling of the defence establishment from the cutting edge of the scientific discipline, which, probably more than any other, has yielded the seminal scientific advances on which revolutionary military development are based. . ." says the study.

The poor performance of industry and DoD in basic physics research may be correlated because each year DoD reimburses industry for some fraction of relevant research through the Independent Research and Development funds. So US industry has invested very little — even of its DoD money — in basic and applied physics. Overall, the study suggests that the recent rise in US defence research funds may not automatically reinvigorate US basic physics.

Deborah Shapley

CORRESPONDENCE

Whose game?

SIR — In his review of J. Maynard Smith's *Evolution and the Theory of Games*, R.C. Lewontin¹ refers to his own "introduction in 1961 of the apparatus of game theory into evolutionary biology". Students of the history of ideas may be interested to know that, about a week after a seminar on this subject given here by Lewontin in 1962, and shortly before R.A. Fisher's death here, the latter also gave us a seminar on the same subject. With the "characteristic modesty" to which Lewontin also refers in his review, Fisher pointed out that not only had he introduced game theory ten years before von Neumann² but he had also discussed it in relation to evolution before Lewontin: this was in a paper that was given to the 30th session of the International Statistical Institute at Stockholm in 1957 and subsequently published twice^{3,4}. I shall leave readers to judge for themselves the importance of these contributions but would like to record the main example that Fisher gave us at the seminar since I think it was never published and may be of widespread application to the explanation of some life cycles that are ecological enigmas. He suggested that the massive production of fruit which occurs infrequently and at random in northern deciduous forests ("mast years") illustrated the principle that, if an opponent (in this case nut-eating animals) cannot be defeated, then it is advantageous to randomize vulnerable activities (nut-production). His biographer⁵ records Fisher's fondness for pigs and, since these animals used to be a major beneficiary of mast years, I suspect that there is a connection.

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On Time defended

SIR — I was interested to read the review by P.W. Atkins in the 11 November issue of *Nature* (p.133) criticizing *On Time* by Michael Shallis, since I have recently read and enjoyed the book. I found the tone of Atkins's remarks derogatory in the extreme.

A point that I would like to make is that scientists of the first category (as defined by Atkins) are so often the blinkered, passive donkeys of their professions who will deny a goodly portion of life's experiences because it is not possible to measure them quantitatively. I imagine that had Atkins been around at the time of Galileo he would have been enthusiastically pouring scorn on the "outrageous" heresy of a heliocentric planetary system for much the same reasons as he scorns Shallis's attempt to look at subject matter which is regarded as "unscientific". It is so easy to be an Atkins and to use these reductionist attitudes to stay safely within the comfortable known behaviour patterns of the

peer group. It is very difficult to be a Shallis and to admit that we do not know it all and that there are areas where we can only wonder and conjecture.

I would like to know what indications Atkins has managed to find in the book that Shallis "disregards the successful continuing progress of modern science". It seems strange that Atkins should carp about this as his own popular lecture on all science rapidly coming to a single all-embracing conclusion, leaving scientists as "keepers of the truth", leaves one with the strong impression that here at least is one scientist who has come to a grinding halt and who one would expect to welcome, not deride, others whom he sees, however wrongly, as being in the same boat.

On Time is a well written, thought-provoking book which deserves better than to have attracted such a destructive onslaught.

V. ANN BUCKLE

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Cometary record

SIR — In my News and Views article on the recovery of Halley's Comet (*Nature* 25 November, p.318), I stated that Halley's Comet (at recovery 11.04 AU away from the Sun) was the most distant comet ever seen, beating the previous record held by Comet Stearn (1927IV). I was wrong and must thank Professor R.A. Lyttleton for pointing out my error. According to S.K. Vsekhsvyatskii (*Physical Characteristics of Comets*, 1927IV was last seen on 13 March 1931 when it was 11.53 AU from the Sun. So Stearn is still the record holder. For Halley to usurp its position we will have to observe it into August 1989.

DAVID W. HUGHES

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Grouping Darwin

SIR — In his autobiography *The Gates of Memory* Sir Geoffrey Keynes¹ revealed that the family he used to illustrate the inheritance of the ABO blood groups in his book *Blood Transfusion*² was his own and his wife's. Charles Darwin was Lady Keynes's grandfather.

Of Charles and Emma Darwin's five children, four were blood-group O and one, George, Lady Keynes's father, was untested. Charles and Emma must therefore each have carried at least one O gene, and of their grandchildren, only George's children could provide further relevant information, since George's blood-type was not determined.

George's wife Maud was blood-group A, and of their four children, two were O (one was Lady Keynes) and two were A. Maud must therefore have been of genotype AO, and George must have carried at least one O gene. In the published genealogy only the two "O" children themselves have children, who therefore provide no further information about George.

The possibilities are therefore that Charles and Emma Darwin might each have been AO, BO or OO, and so might their son George. For each of the nine possible genotype-pairs for Charles and Emma we can compute the

probability that they had four OO children and a fifth who on marrying an AO had two O and two A children (and hence certainly carried on O gene).

The calculation of these probabilities is entirely straightforward and I therefore omit the details; it should be noted that because the only person other than Charles and Emma entering the genealogy except as a child is of known genotype (Maud, who is AO), the probabilities are not functions of the unknown gene frequencies in the general population. This is, of course, unusual in this type of problem.

The resulting probabilities expressed relatively are the likelihoods³ of the several hypotheses:

Relative likelihoods of Charles and Emma Darwin's genotypes

OO × OO	16,384
OO × AO or AO × OO	800
OO × BO or BO × OO	544
AO × AO	43
BO × BO	19
AO × BO or BO × AO	26

It will be seen that it is overwhelmingly likely that both Charles and Emma Darwin were blood-group O, the next most likely hypotheses being over 20 times less likely.

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Acidity and alkalinity

SIR — "Acidity" is an awkward term when used as a heading for discussion of effects brought about by varying hydrogen ion concentration. It is limiting in first appearances because it also doubles in general usage as a term implying only the acid end of the scale. The older term "hydrogen ion concentration" does not itself convey the concept we are often trying to put across; that which we neatly sum up in the jargon term "pH".

When we are heading manuscript sections for variables such as "temperature", "pressure" and so on, the heading word "pH" seems too short. We tend to get around the problem by writing "effect of pH". Some manoeuvring is also required to ensure that we do not have pH as the first word of a sentence, since we cannot capitalize it.

For the parallel concept of oxidation and reduction we rarely use the symbol "E", meaning the oxidation-reduction potential, in a sentence. Certainly the phrase "transferable electron potential" is an unusual heading. The convenient word "redox" has come into common use.

Therefore, by analogy, I propose the term "alkacidity" as a convenient word to express an important idea in brief.

PETER MEREDITH

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NEWS AND VIEWS

A superfast new pulsar

from David J. Helfand

FIFTEEN years ago last autumn, Morse-code-like signals pulsing with extreme regularity were received at a Cambridge radio telescope — pulsars had been discovered. Although the first such object was dubbed an LGM (for Little Green Man), a total of four such sources had been identified by the time the discovery was announced in February 1968 and the signals were soon explained, without recourse to the presence of extraterrestrial intelligence, as lighthouse-like flashes from highly magnetized rotating neutron stars. With nearly 350 objects now catalogued, it might seem surprising that the detection of a new pulsar would generate much excitement. But PSR1937+214, whose discovery is reported by D. Backer *et al.* on page 615 of this issue of *Nature*, is not typical; it is rotating at an astonishing 642 times a second and may well represent the first of a whole new class of objects.

The story of the discovery is one of perseverance combined with a dash of good luck. The source first appeared in the fourth Cambridge catalogue of radio emitters as 4C21.53. Its peculiarity was first noted in 1972. Using the Cambridge interplanetary scintillation telescope with which pulsars were discovered, A. Readhead and A. Hewish reported¹ that the source 'twinkled' in the interplanetary medium, unusual behaviour for an object lying in the plane of the Galaxy where interstellar material generally broadens the apparent angular diameter of compact sources and destroys the IPS modulation. Backer's attention was drawn to the source in 1979 following a report² that it had a steeply rising spectrum at low radio frequencies which, along with the very small diameter implied by the IPS measurement, is a characteristic of radio pulsars. In perusing the literature, he noticed that 4C21.53 was roughly coincident with a diffuse region of emission with a contrasting flat radio spectrum. This combination of a scintillating, steep-spectrum source and a diffuse flat-spectrum region was strikingly reminiscent of the Crab Nebula and its embedded 33 ms pulsar, the youngest and fastest pulsar then known.

This area of the sky had been searched for pulsars in 1973 by R. Hulse and J. Taylor, then of the University of Massachusetts, using an extremely sensitive system at the Arecibo Observatory 1,000-foot radio telescope. As in most of the large-scale pulsar searches conducted to date, the workers would have found it impossible to detect a new pulsar with a period significantly shorter than that of the Crab since the threshold period for the search was ~ 30 ms. This constraint is usually dictated by limitation on computer time, but this had not been deemed a serious problem: there are no other objects like the Crab Nebula within the search range ($\sim 10^4$ light yr), and it was thus assumed that there were no younger and faster pulsars to be found. The assumption, that all fast pulsars are young, may have been the critical flaw in the argument.

In 1979 Backer conducted a search for a shorter-period source in 4C21.53 at both the Owens Valley Radio Observatory and at Arecibo without success. His interest persisted, however, and, in November, some tantalizing earlier results from S. Kulkarni and C. Heiles were followed up with a search for interstellar scintillation which has, to date, only been observed for pulsars. Deep modulation was found and the following day, the periodicity of 1.5578 ms was confirmed.

The source is remarkable in many ways. The rotation rate is within a factor of about 3 of centrifugal break-up. The star's surface is moving at ~ 20 per cent of the speed of light and the rotational kinetic energy of the object is nearly 10^{52} ergs, comparable with the entire energy output of a supernova explosion. Furthermore, this superfast pulsar shows no evidence of the

encircling supernova remnant expected to accompany the creation of such an object.

A measurement of the pulsar's slowdown rate is critical in addressing several important questions including the object's age, origin and energy loss mechanism (for example, gravitational waves versus magnetic dipole radiation and energetic particles). The limit quoted by Backer *et al.* implies a period derivative $\dot{P} < 10^{-15}$ seconds per second (s s^{-1}), a factor of several hundred smaller than the Crab. J. Taylor of Princeton University has recently taken his equipment used in monitoring the binary pulsar to Arecibo and has already lowered this limit substantially in a week's observation. It thus appears that, despite its huge store of rotational kinetic energy, the pulsar's energy output is quite small. Optical observations, also reported in this issue, and X-ray data taken with the Einstein Observatory by R. Becker suggest that the high-energy emission from this object both from within the magnetospheric (that is, pulsed) and from a pulsar-powered synchrotron nebula is several orders of magnitude less than that from the much slower Crab pulsar. These results will dampen the initial excitement caused by the discovery among the experimental general relativity community, where the early report³ of a high spindown rate led to quickly drawn plans for gravity wave detectors tuned to the pulsar frequency.

Backer *et al.* suggest that the pulsar and the nearby diffuse source (now known to be an HII region) may be associated, with the latter excited by an escaped binary companion of the fast pulsar. From the separation of the two sources and using a typical pulsar velocity, they infer an age for the pulsar of $\sim 10^4$ yr. The case is far from compelling, however. More recently, A. Alpar, M. Ruderman and J. Shaham of Columbia University, together with A. Cheng from Rutgers University, have proposed a scenario in which the neutron star is billions of years old and was reactivated as a pulsar after having been spun up by accretion from a companion. In a letter to appear in the next issue of *Nature* they suggest that the source is the remnant of a galactic bulge X-ray binary system. The standard scenario for bulge sources consists of an evolving low-mass star spilling matter onto a neutron star with a very low magnetic field. For such an object, the Alfvén radius at which the magnetic field dominates the inflowing matter is very close to the stellar surface, allowing for a large transfer of angular momentum to the star, spinning it up to millisecond periods. The ultimate spin rate is a function principally of the field strength and the mass transfer rate. Once the accretion stops as a result of the dissipation or ejection of the companion, the pulsar will begin to spin down again. But with its low field, the spindown time will be very long, preserving a population of old rapidly spinning pulsars.

The strength of this picture is that it offers a prediction of the current magnetic field strength and, thus, of the period derivative for such a source. The model gives the right answer for $\dot{P}$ for each of the three known binary pulsars, all of which have anomalously low spindown rates for their short periods. For 4C21.53, the prediction of $\dot{P} < 5 \times 10^{-20} \text{ s s}^{-1}$ will be testable within a few months.

Many questions remain to be answered about this exciting new object and searches for other examples are already being planned. Future studies will focus on what such sources can tell us about the mechanism of pulse emission, neutron star interiors, and the origin and evolution of pulsars. At the moment, though, 4C21.53 serves as a reminder of the richness of the physical Universe in which each unexplored corner of parameter space holds a new mystery for the astrophysicist to ponder.

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'Lucy' jilted?

from M.H. Day

WHAT'S a girl to do if her date lets her down? This question often has to be answered by girls who have waited in vain — but the question of interest here is has 'Lucy' (or to be more precise her slightly older 'relations') waited 3.3 or 3.6 million years before her date with Don Johanson, Yves Coppens and Maurice Taieb? These three, the leaders of the French/American expedition to the Hadar region of Ethiopia, have been responsible for the recovery of the early hominid material, known collectively as *Australopithecus afarensis*, from deposits that were originally said to range in age from 2.6 to 3.1 million years before the present. The famous Lucy is said to be part of that species, as are Mary Leakey's earlier Laetoli hominids from Tanzania¹. Earlier this year, however, it was suggested that the dates of the basalts from the Hadar series had been underestimated by as much as 300,000–600,000 years, bringing the earliest date of the Hadar site much closer to that of Laetoli, the site from which the *A. afarensis* type specimen was chosen², this species being regarded by some as representing the common ancestor of the hominid stem.

An article³ in this issue of *Nature* (p.631) now questions this revision of the lower end of the temporal range of the Hadar hominids on grounds of stratigraphical correlation between the Hadar Formation and sites in the Lake Turkana Basin, together with faunal studies that may be consistent with the younger rather than the older age for the bottom of the Hadar sequence. F.H. Brown reports the results of microprobe and X-ray fluorescence analysis of a group of volcanic tuffs from the Koobi Fora Formation (Tulu Bor), the Shungura Formation (Tuff B) and the Usno Formation (Tuff U-10). All these air fall tuffs are the result of volcanic eruptions and all are found in the Lake Turkana Basin of northern Kenya and southern Ethiopia. Further north in Ethiopia, the Sidi Hakoma Tuff has also been sampled from the Hadar site and added to the comparisons. The microprobe analyses of major and minor elements and the X-ray fluorescence analyses of minor and trace elements show remarkable correspondence in all of the tuffs examined and lead to the view that all were the result of broadly contemporaneous events and thus may indicate isochronous litho-stratigraphical horizons. This is said to produce a 'direct lithostratigraphical tie' between the Hadar Formation and the Lake Turkana region which can help to date the fossil faunas of the two areas,

including the hominids.

The palaeomagnetic evidence shows that the three tuffs from the Lake Turkana basin are all of normal polarity and they are thought to lie in the Gauss Normal Epoch, having therefore an age of between 3.17 and 3.42 million years. The Sidi Hakoma Tuff also has a normal polarity. The two reversed zones above the Sidi Hakoma Tuff and their equivalents in the Shungura and Usno Formations have been regarded as the Mammoth and Kaena Events.

Potassium-argon (K/Ar) and fission track dates from the Hadar Formation are said to be consistent with those identifications with one interesting exception, the 3.6 Myr date for the Type B sample of the Kadada Moumou Basalt (KMB)². The date for this layer has been revised by Brown³ to 3.12 Myr by applying a 3.5 per cent upward 'correction' to the 3.01 Myr date for the original Type A sample of the KMB, so that the reversed polarity of the KMB layer can sit conveniently in the Mammoth Event rather than be attributed to the underlying Gilbert reversal as it would be at 3.6 Myr. In the Lake Turkana Basin the palaeomagnetic identifications also apparently accord with the K/Ar dates on the tuffs, with the exception of the date of Tuff B10 of the Shungura Formation which is said to be 'too old' at 3.75 Myr. This tuff is one of the uppermost units of the Shungura Formation and lies just below Unit B10, which produced most of the fauna from Member B referred to in a second paper⁴ published in this issue of *Nature* (p.633) that deals with the age of the Hadar Formation.

In these days of 'high-tech' science, there is something delightfully anachronistic about consulting an assemblage of fossil bones to support or disprove a numerical age produced by radiometric means. This was exactly what happened at Koobi Fora, however, where it took the 'pig clock'^{5,6} to sort out the correct date for the KBS Tuff. Biostratigraphy, therefore, still has an important part to play in geochronology since its cumulative effect may also be enhanced by comparisons from several adjacent sites. In the paper by Boaz, Howell and McCrossin (p.633), two large-mammal faunal lists, one from the Usno Formation (Lower B) and the other from Shungura (B10), have been compared with the Hadar assemblage and with each other by means of faunal similarity coefficients.

The Usno and Shungura comparative assemblages are given 'best-fit' K/Ar and palaeomagnetic dates of 2.92 and 3.15 Myr respectively and thus a time difference of about 0.2 Myr. The results show firstly that the Usno and Shungura assemblages are nearer to each other than either is to Hadar and secondly that by any test the Hadar and Usno faunas are closer than the Hadar and Shungura B10 faunas, suggesting that the Hadar fauna is closer to the older of the two dates. Unfortunately, sites such as Laetoli, Kanapoi, Mursi and Ekora, with a

range of 3.2–4.0 Myr, cannot be compared with Hadar at present since their inventories are not complete. The faunal evidence, as far as it goes, therefore, seems to be consistent with the date for the Hadar site put forward by Brown, yet a pre-Usno date for Hadar is prudently not rejected. It seems that the correlative stratigraphical and faunal case for a younger lower limit for the Hadar site is interesting but not compelling.

What, then, would be the significance of this proposed revision (or perhaps reversion is a more appropriate word) of the dating of the Hadar site for hominid evolution? All seem agreed that the bulk of the Hadar hominids are older than 2.88 Myr. The lower limit of the range at Hadar is either 3.12 or > 3.6 Myr, a difference of 400,000 years or more with a possible span of 240,000–720,000 years at this site. This means that the Hadar sample of hominids is drawn from either a quarter or nearly three quarters of a million years; this must colour the views of those who would tackle the question of whether the Hadar sample is homogeneous and monospecific. Indeed, in relation to the Laetoli site, a gap of 370,000 years has re-opened in the evolutionary history of *A. afarensis*.

As well as the correctness of including the Laetoli hominid material in the same specific category as that of the Hadar sample, the question of the age of the Makapansgat site in the Republic of South Africa now begins to become relevant. A younger date for Hadar, if proven, coupled with an older date for Makapansgat^{7,8}, could make it uncertain that Makapansgat *Australopithecus africanus* is more recent than Hadar *Australopithecus afarensis*. Laetoli is, of course, still older than either site; but if the specific unity of the Hadar *A. afarensis* sample or the inclusion of the Laetoli hominids in that sample is shown to be incorrect, then *A. afarensis* could be a stem form in name alone. The folly of selecting a type specimen both geographically and temporally separated from the largest and best fossil sample would then be exposed once more.

To let a girl down on her date is bad enough, but to get her name wrong when you do arrive would be intolerable!

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CORRIGENDUM

In the *News and Views* article 'Human monoclonal antibodies' (*Nature* **300**, 316; 1982) D. Crawford's affiliation was given as the Imperial Cancer Research Fund Laboratories, London. She is in fact at the Department of Haematology, University College London.

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Mouse and supermouse

from Jeffrey G. Williams

THE mice displayed on the cover of this week's *Nature* are littermates but one has a bodyweight almost twice that of its sibling. They differ in that the larger mouse carries a hybrid gene containing the promoter of the mouse metallothionein-1 gene fused to the structural gene for rat growth hormone (see p.611). The 'transgenic' mouse was produced by injecting the hybrid gene into the pronucleus of a fertilized egg. Mice carrying the gene display very high levels of the fusion mRNA in their liver, high levels of growth hormone in their serum and an elevated growth rate. This is the first time that genetic engineering has been used to alter the phenotype of an animal in such a profound manner and is the culmination of a series of elegant experiments performed by Palmiter, Brinster and their colleagues over the past few years. There are almost certain to be great scientific and practical benefits from the application of genetic engineering techniques to animals. It is essential, however, to stress that there are limitations on what can be done — and more important, on what is worth doing.

When cloned genes are introduced into fertilized eggs by micro-injection a proportion of the mice that develop will carry the gene. The injected genes become chromosomally integrated but they are not found at the normal, nor even at a unique, location and the number of gene copies per cell also varies from animal to animal. The mice that develop from the injected eggs may also be chimaeras in which only some of the cells contain foreign DNA. However, a proportion of mice do transmit the newly acquired gene to their progeny and it is thus possible to produce mice containing the gene at a defined position in every cell.

The product of the mouse metallothionein-1 gene is a small cysteine rich polypeptide that binds heavy metals and is thought to be involved in zinc homeostasis and resistance to heavy metal toxicity. It is present in most tissues of the mouse, but the highest levels are found in liver. Transcription is inducible by heavy metals and glucocorticoids. Experiments reported earlier this year (Brinster *et al.* *Nature* **296**, 39; 1982) used a hybrid gene to establish that the sequence elements required for heavy metal inducibility lie within a stretch of DNA ninety nucleotides upstream of the start point of transcription. The hybrid gene contains a segment of the 5' non-coding region and upstream sequences derived from the metallothionein-1 gene. Both were fused into the 5' non-coding region of the structural gene for herpes virus thymidine kinase to provide a readily assayable enzymatic activity. The hybrid

gene was injected into fertilized mouse eggs which were incubated with cadmium as an inducer and then assayed for thymidine kinase activity.

The same fusion gene was used in two subsequent studies in which micro-injected fertilized eggs were re-implanted into foster mothers (Brinster *et al.* *Cell* **27**, 223; 1981; Palmiter *et al.* *Cell* **29**, 701; 1982). A high proportion of mice which carried the fusion gene expressed thymidine kinase in the liver. The activity was inducible with heavy metals but not with glucocorticoid hormones. For unknown reasons, in the progeny derived from these mice the expression of thymidine kinase was diminished or abolished in some cases and enhanced relative to the parent in others.

The metallothionein-growth hormone fusion gene was constructed using exactly the same principle as the *tk* fusion gene, with the genes being joined in their 5' non-coding regions. The mice contained variable numbers of copies of the gene and, with only one exception, the mice with the highest growth rate had the most copies of the gene. The mice were fed zinc to induce transcription of the fusion gene but this may not have been necessary. Most of the mice containing the gene were larger than normal before being fed zinc, and one animal which was removed from the zinc diet continued to grow at an accelerated rate. In mice with the highest copy number of the gene the level of growth hormone production was between 100 and 800 times the normal level. The serum concentration in one of the mice actually exceeded 100 $\mu\text{g ml}^{-1}$.

Thus, aside from the variability of expression in second generation mice and the lack of response to glucocorticoids, results with the metallothionein promoter are highly encouraging. Sadly for the developmental biologist, however, this is not the case for globin genes. Although transcripts have been detected in muscle tissue from one mouse line carrying the rabbit β -globin gene, neither the rabbit β -globin gene, nor a fusion gene containing the 5' half of the mouse β -globin gene and the 3' half of a human β -globin gene are expressed in the blood cells of seven mouse lines carrying the rabbit gene and two lines carrying the fusion gene (Frank Constantini and Elizabeth Lacy, personal communication). This difference in behaviour of the β -globin genes and the metallothionein gene in transgenic mice may be a reflection of a fundamental difference between the two genes. Globin genes are expressed only in cells of the erythroid lineage and only at a specific stage in development. While expression of

the metallothionein-1 gene varies from tissue to tissue, most tissues express the gene at some level. Thus metallothionein might almost be regarded as a housekeeping gene which displays tissue specific variation in the extent of expression. This in no way detracts from the usefulness of this promoter in obtaining high levels of expression of any gene to which it is suitably fused, and this is powerfully demonstrated by the growth hormone study.

The authors discuss a number of implications of their results. The elevation of serum protein levels by these means could provide a way of studying hormone action a way of accelerating animal growth, a method of producing valuable gene products and a means of correcting genetic disease. Before considering these last three applications individually there is one point which should be made. They all rely to a greater or lesser extent on the transmission through the germ line of the ability to express the gene. However, the metallothionein-1 thymidine kinase fusion gene was sometimes not expressed in the offspring of mice which were themselves expressing the gene. If the metallothionein-growth hormone fusion gene behaves similarly this phenomenon will require further investigation.

The benefits which might accrue from a shorter production time seem a sufficient spur to ensure that fusion genes of this kind will be used to accelerate the growth of livestock animals. Also, the novel concept of genetic farming — the use of animals to produce large amounts of commercially important proteins — may be applied to the production of proteins which require secondary modifications not correctly made in yeast or other eukaryotic host cells. Finally, this achievement once again brings into question the potential of this technology as a means of correcting genetic disease. It will have become clear that such an approach will only be applicable where a genetic disorder may be prevented by simply elevating the serum concentration of a particular protein. In order to correct a genetic disorder such as a thalassemia by manipulation of the embryo, expression of the introduced gene would probably have to be limited to cells of the erythroid lineage and this is not at present achievable. Also, for reasons which have been presented in a recent review in this journal (Williamson, R. *Nature* **298**, 416; 1982) it seems exceedingly unlikely that there will ever be a clinical requirement for such therapy to be applied to the embryo. Control sequences such as the metallothionein promoter may, however, prove useful in developing methods of correcting gene disorders in the somatic cells of affected patients.

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Hypertension and inhibition of the sodium pump: a strong link but in which chain?

from Ian M. Glynn and T. J. Rink

THE experiments reported in this issue of *Nature* (p.650) by Hamlyn and his colleagues¹ from Baltimore, showing that the blood from patients with high blood pressure contains an inhibitor of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$, are welcome for their cogency. Similar claims have been made before^{2,3}, but always on the basis of evidence too indirect to be wholly convincing in a field where direct testing is feasible. The direct test has now been made and, though the effects are small, the answer seems clear. The blood plasma of patients with essential hypertension, that is, with a permanently raised arterial blood pressure not secondary to any known cause, is more effective at inhibiting a partially purified $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ (the enzyme that pumps sodium ions across cell membranes in exchange for potassium ions) from dog kidney than is plasma from normal subjects; and the higher the blood pressure the more inhibitory the plasma tends to be. Furthermore, since plasma from most normal subjects is slightly inhibitory, the inhibitor may be of physiological as well as pathological interest.

The crucial question is whether correlation implies causation: does the inhibitor have a role in the aetiology of essential hypertension, and, if it has, what is that role?

There is no doubt that the new results fit well with the notion that essential hypertension is a disease in which the inability of the kidneys to cope normally with salt and water loads is compensated for, not only by a rise in blood pressure⁴⁻⁶, but also by an increase in the plasma concentration of a (hypothetical) hormone that promotes salt and water excretion by inhibiting sodium reabsorption in the kidney tubules⁷⁻⁹. The circulating $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ inhibitor could well serve as the hypothetical hormone, though there is so far no evidence that it does in fact inhibit sodium reabsorption in the kidney tubules or that it has a natriuretic (promoting salt excretion) effect.

The results also fit well with the hypothesis that the narrowing of the arterioles — which everyone agrees is the immediate cause of the raised arterial blood pressure in established essential hypertension — represents a secondary effect of the increased level of the hypothetical sodium-transport-inhibiting hormone. The suggestion is that inhibition

of the membrane sodium pump in the arteriolar smooth muscle cells leads to a rise in intracellular sodium concentration, which, by the action of a $\text{Na}^+ - \text{Ca}^{2+}$ exchange mechanism, increases intracellular calcium concentration and hence contractility¹⁰.

Attractive though this hypothesis may be, it is still only a hypothesis. There is evidence that inhibition of the sodium pump in smooth muscle, by a reduction in extracellular potassium or by high concentrations of cardiac glycosides, can lead to an increase in arteriolar resistance in the rat¹¹, but it is not clear that the circulating inhibitor reported by Hamlyn *et al.* would have a similar effect, or, if it had, that the effect would be maintained. It is perhaps ominous that an almost identical hypothesis^{12,13} proposed in the late 1960s to explain the ability of cardiac glycosides to increase the force of contraction of the heart still remains controversial. And though it is comforting to have a plausible hormonal explanation of the paradox that salt and water retention can lead to hypertension associated with a normal cardiac output and increased arteriolar resistance, the explanation proposed may not be sufficient or even correct. The consensus of opinion seems to be that in patients with coarctation of the aorta (a pathological narrowing of the aorta above the level of the renal arteries), the arterial blood pressure is high in regions above the coarctation and roughly normal in regions below it — in other words the pressure is adjusted to suit the kidneys — yet the blood flow through the arterioles is normal in both regions^{4,6,14}. This implies that the calibre of the arterioles is reduced in the upper half of the body but not in the lower, a fact that is not easily explained if the reduced calibre of the arterioles is caused by the action of a circulating agent on the smooth muscle of their walls.

Strong evidence that a circulating agent can be involved in the genesis of hypertension comes from experiments in which rats of different strains had their skin and subcutaneous tissue joined so that substances could exchange between the bloodstreams. When rats from a strain that develop hypertension on a high-salt diet had their circulation linked to rats that did not respond in this way, the originally unresponsive rats rapidly developed hypertension when fed salt¹⁵. These experiments have been interpreted to suggest that the salt-sensitive rat on a high-salt diet produces a circulating natriuretic hormone which increases the contractility of the smooth muscle of the arterioles⁹. That is not the only possible interpretation, however; it is at least as likely that the effect

of the transferred circulating factor is to make the kidney of the originally salt-resistant rat less able to excrete salt and water, and that the hypertension arises secondarily. It would be interesting to see whether plasma from the salt-fed, salt-sensitive rat behaves like plasma from hypertensive patients in inhibiting $(\text{Na}^+ + \text{K}^+)\text{ATPase}$.

Other questions that must now be asked concern the relation between the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ inhibitor demonstrated by Hamlyn *et al.* and (1) the substances present in the plasma of hypertensive patients that can increase the vascular reactivity of rats to noradrenaline and angiotensin¹⁶, (2) the high- and low-molecular weight natriuretic substances that have been extracted from the urine of salt-loaded subjects¹⁷, (3) the natriuretic substance found in the urine of normal men immersed up to the neck in water at 34.5°C (ref. 18), and (4) the natriuretic and $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ -inhibiting substance found in the plasma of volume-expanded rats¹⁹. It will also be necessary to consider to what extent the presence of circulating $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ inhibitor can explain the numerous and sometimes contradictory reports of abnormalities in sodium, potassium and lithium transport in the red blood cells and white blood cells of patients with hypertension and of their relatives with normal blood pressure.

Presumably the first priority of the group at Baltimore will be to characterize their inhibitor. They have already shown that it is heat-stable, that it does not cross-react with antisera to cardiac glycosides and that it acts rapidly even at concentrations at which the maximal effect is small. It should not be difficult to gain a rough idea of its molecular weight, or to determine whether or not it is organic. More precise identification will be more difficult, but it must be an advantage that the assay is so simple. When the inhibitor is identified and can be prepared in quantity, it should be possible to determine whether it can produce a maintained narrowing of

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arterioles, whether it can promote salt excretion by the kidney and whether the kidneys of hypertensive animals or patients are less sensitive to it than are the kidneys of

normal animals or patients. The answers to these questions should define the role of the inhibitor in the aetiology of hypertension — if indeed it has one. □

industry from Germany in the first century AD but it has not yet been recognized archaeologically although it would have left debris.

Before 500 BC coastal working of salt in regions where boiling was necessary (the Atlantic and North Seaboards) was rare^{2,3} and only around the Mediterranean Sea, where solar heat was sufficient to evaporate the brine, does sea-salt seem to have been important⁴. Routes inland, such as the 'Via Salaria' in Tuscany (from near Ostia to the Sabine Hills), existed from at least the fifth century BC, but there is no evidence that sea-salt was carried up into central Europe.

North of the Alps, some inland sources of salt were used in the early part of the first millennium, as they had been in the second millennium, and the three areas with major reserves of salt, shown in the map below left, were increasingly exploited. Estimates of salt production show that it was in excess of local needs and that wide regions were being supplied^{3,5}.

In Austria, where names like HALLstatt and HALlein-Dürnberg preserve references to salt, thick veins of common salt were followed down for more than 400 m by skillfully constructed shafts and galleries. These, similar to contemporary Alpine coppermines, must have been the work of experienced miners^{6,7}. They were especially active between 1000 and 400 BC when the 'eastern workings' produced thousands of kilogrammes of salt in heart-shaped slabs weighing up to 40 kg each. Nearby contemporary cemeteries are very rich in metal objects, especially jewellery, of types found within 200 km of the production sites and may well reflect the wealth resulting from a long-distance trade in salt slabs. The highly skilled miners appear to have worked only in the summer in the high, isolated alpine valleys at Hallstatt, and caravans of pack animals or porters would have been necessary to carry away the slabs. The mines appear to have gone out of use after 400 BC. A distant analogy with the Saharan fourteenth to sixteenth century AD saltmines at Taghaza,

The prehistoric salt trade in Europe

from John Alexander

SUFFICIENT archaeological evidence has now accumulated for a picture of the winning and trading of common salt in prehistoric Europe to emerge. North of the Alps several big inland deposits were exploited and traded over long distances in the years before 500 BC. In the Mediterranean coastlands sea-salt was used, but further north, where boiling was necessary, this was not at first common. After 500 BC the inland suppliers disappeared and production moved to the coasts, perhaps as a result of the changes brought about by the mass migration of peoples at that time.

Common salt is not just a luxury for human communities with largely cereal diets, but is an essential. The salt received from flesh, blood and urine is insufficient for dietary needs. Salt may also be required for preserving flesh (including fish) and hides, or for religious purposes. Since substantial deposits of salt are rare in Europe and many regions are remote from seashores, salt was traded over long distances, as is known from the earliest written records (the last few centuries BC). Detailed knowledge of the industry in earlier prehistoric times has always been difficult to come by since the traded salt does not survive, but much recent excavation at the production centres makes it possible to find out when they were exploited, whilst a study of traded metal objects suggests the routes used for salt-trading¹.

Common salt is obtainable in quantity

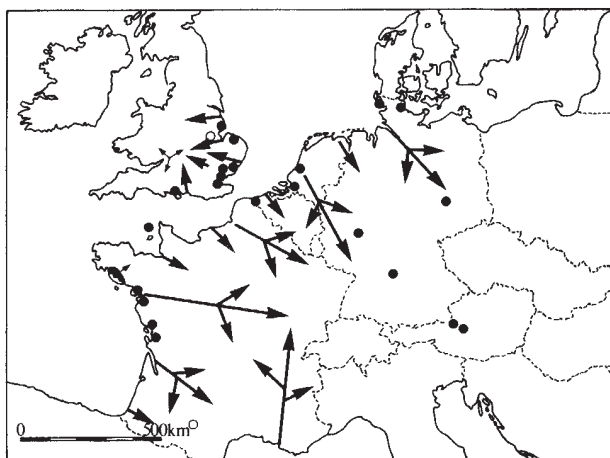
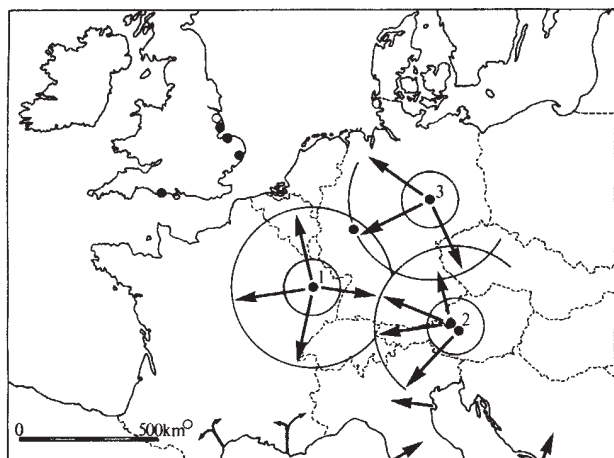
from four kinds of source — sea-salt, rock salt, saline springs and salt-rich plants — all of which were present and utilized in Europe. Sea (or bay) salt is only won with ease on flat coasts where advantage can be taken of tidal variation. In the Mediterranean coastlands the work is relatively easy as solar heat is sufficient to evaporate the brine. Elsewhere boiling is essential and the collection of fuel and the making of vessels are major additions to the work. The debris from the boiling process (broken vessels, burnt earth and charcoal) can be recognized and dated, as in the 'red hills' of Essex.

Rock (or halite) salt can only be obtained in quantity by mining, but needs little further processing. In the few localities where mining occurred, as in central Austria, shafts and galleries dug along the seams have been found and dated.

Brine from inland saline springs, or from the salt-impregnated earth around them (from which the salt must be dissolved and cleansed before boiling) will leave debris similar to the seaside boiling-sites and can, as in the Saale Basin in Germany, be recognized and dated.

Salt-rich plants can be burnt and their salt dissolved and evaporated by boiling. Tacitus reported the existence of the

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The suggested pattern of (left) salt production in western and central Europe before 500 BC: (1) Moselle Basin, (2) Salzkammergut, (3) Saale Basin; and (right) after 500 BC (after ref. 2).

where the slabs were carried over 1,000 km by regular caravans, is interesting^{8,9}.

At the same time a different kind of large-scale exploitation was being practised in the Saale Basin in Saxony. Here around HALLE, salt springs over some 200 km² were exploited. The brine was boiled in batteries of distinctive pottery vessels and mounds of debris five metres high have been found. As in Austria, there is an absence of remains after 400 BC, suggesting that production ceased for some centuries^{10,11}. Very large numbers of conical lumps of hard salt weighing several kilogrammes were produced and were probably dispersed over a wide area, perhaps several thousand square kilometres. The mechanism for production and dispersal may well have differed from that in Austria, since no great expertise would have been necessary, although the labour required suggests considerable organization. A distant analogy may be seen in the brine springs at Uvinza in Tanzania which are scattered over 30 km² and were worked seasonally by up to 20,000 people in the nineteenth century AD¹².

A third centre, worked on a similarly large scale in the same centuries, lay in eastern France among the brine springs of the Moselle valley near Seille. Here, over some 200 hectares, debris from brine boiling in great banks up to 17 m high are found. Once again production seems to have stopped after 400 BC. Production on this scale suggests that the salt served the needs of a wide area^{13,14}.

No other inland centres as large as these three have been found and it seems likely, in the absence of extensive coastal salt-boilings, that they supplied most of the North European Plain with salt (see map on left). Long-distance trading, as in Africa, was accompanied by trade in more durable, especially metal, objects²⁰.

A major change in salt production seems to have taken place after 400 BC. Large-

scale working at inland centres stopped while the number of coastal brine-boiling sites greatly increased. The new sites appeared on the Atlantic coasts of France^{15,16}, Belgium¹⁹ and southern England¹⁷ in numbers too great merely to satisfy the needs of coastal communities (see map on bottom right of p.577). There was probably a salt-trade inland, by boat up the rivers, since there are many boiling sites near estuaries. Trading of this kind penetrated some 300 km into the hinterland of West Africa in the nineteenth century AD¹⁸.

That there was some connection between the decline of the inland centres and the rise

of the coastal ones in the fourth to second centuries BC seems likely, but what brought about the change is not yet clear. Those centuries saw great changes in central Europe, with the migration of large numbers of Celtic-speaking peoples into Italy and the Balkans and the development of the La Tène culture. These may well have interrupted patterns of control and trade and been coupled with the exhaustion of supplies of firewood and the need for deep, easily flooded mines. Coastal production in areas with greater resources of firewood and with less opportunity for centralized controls may have taken advantage of this.

Ball lightning wild and wonderful

from Martin A. Uman

BURBIDGE and Robertson report in this issue of *Nature* (p.623) the unusual observation of a liquid-like 'blob' entering a house under a door and moving along the floor immediately after lightning struck extremely close by. Because of the association with lightning, it is tempting to classify the 'blob' as ball lightning, although it represents an extremum in the spectrum of observations of that phenomenon for it had a very low luminosity, lacked a well defined shape and was not free-floating.

Reports of ball lightning can be found in both ancient and modern literature and the similarity in described size, colour, smell, duration, sound at demise and physical damage lends overwhelming support to the view that ball lightning is a real phenomenon (rather than a visual after-image or a psychological aberration). Nevertheless, no adequate laboratory simulation of ball lightning has been achieved, although similar phenomena are occasionally generated by accident in high-current electric machinery.

Most ball lightnings are spherical or oval, between the size of a marble and a basketball, exhibit a faint glow and last a few seconds. They appear to float while moving horizontally and can occur inside houses and all-metal airplanes as well as out-of-doors. There may well be more than one kind of ball lightning. For example, the bright spheres which hang high in the air and are seen at considerable distances would seem different from the typical ball lightning described above. On the other hand, these very bright, relatively long-lasting and apparently large balls could represent the upper end of a scale of phenomena in which the 'blob' or 'flow of light' seen by the New Zealand observers, with its low luminosity, lack of well defined shape and location at ground level, could represent the bottom.

While Burbidge and Robertson's observations are of considerable interest,

the idea they put forward to explain the motion of the blob — the existence of free magnetic dipoles or permanent current loops in the blob that interact with the magnetic field gradient — would appear farfetched. How could a current loop exist for 15 seconds in a liquid or gas near ambient temperature and pressure? What available liquid or gaseous material could have permanent magnetic dipoles? It is, nevertheless, interesting to put numbers in equation (2) in Burbidge and Robertson's paper. With the horizontal gradients in the vertical field given in Fig. 1 a horizontal force of about 10⁻⁵ Newtons will be produced by an assumed current of 10 A flowing around the perimeter of the blob. The blob must be slightly more dense than air. If it were less, it would rise. If it were much more, it would appear more like a 'puddle' than a 'blob'. The drag force of the ambient air on such a blob, for the observed velocity of 0.25 m s⁻¹, is about the same as the magnetic force for the assumed 10 A current. Thus, for that value of current, the two forces balance to yield the observed blob velocity. If the vertical gradient in the horizontal field were such as to produce a vertically upward magnetic force of about the same magnitude as the horizontal force, the blob would be levitated if its density were less than 1 per cent greater than air. Otherwise, the magnetic force would be less than the gravitational force, and the blob would remain on the ground. It is worth noting that a 10 A current flowing in an air arc or in an air arc contaminated by vapours of solids produces a level of light output that is painful to look at.

Finally, we should address the question of just what is a ball lightning. There is no consensus! *Nature* is probably the primary publication source for unconventional (if there are truly conventional) ball lightning

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observations and theories. Many of these theories are improbable but do make for interesting reading. A few examples follow. Wooding (Laser Analogue to Ball Lightning, *Nature* **239**, 934; 1972) would have the lightning act as a laser to produce a high-power optical pulse which would ablate a solid surface producing a plasma vortex ring (the ball). The laser radiation would escape at points along the lightning channel when it zigged or zagged. Altschuler, House and Hildner (Is Ball Lightning a Nuclear Phenomenon? *Nature* **228**, 545; 1970) suggest that high-energy protons from lightning collide with air molecules and cause nuclear reactions which produce a high-energy ball. They do note, however, that being near such balls could result in radiation sickness or worse. Ashby and Whitehead (Is Ball Lightning

Caused by Antimatter Meteorites? *Nature* **230**, 180; 1971) report the observation of photons at the energy corresponding to position annihilation, and suggest micrometre-size antimatter meteorites as the source for ball lightning. Crawford (Antimatter and Ball Lightning, *Nature* **239**, 395; 1972) has interpreted the measurements of Ashby and Whitehead to suggest that ball lightning is due to the photons produced when positrons in cosmic ray showers (EAS) come to rest and are annihilated.

Ball lightning is a wild and wonderful subject for study. The interested reader can best be introduced to the available theories and experimental data by reading the book by J.D. Barry *Ball Lightning and Bead Lightning* Plenum Press, New York, 1980). □

are primed by the T7 gene 4 protein which could use its associated helicase activity to unwind DNA, thus exposing primase sites (Richardson). In T4 the virus-coded primase and helicase are different proteins which interact during replication (Alberts, UCSF Medical School).

Studies of replication in eukaryotes have lagged far behind, but promising experimental systems are now emerging for studying several aspects of the process including RNA priming. Thus Conaway (Stanford Medical School) reported that the primase activity from *Drosophila* copurifies with DNA polymerase α , the polymerase primarily responsible for replication in eukaryotes.

A clear exception to the rule of RNA priming is provided by adenovirus, and a controversy has existed over whether the DNA polymerase which replicates adenovirus DNA is α or γ . This appears to have been resolved by the finding that it is a separate polymerase encoded by the virus itself (Hurwitz, Albert Einstein College, New York). The polymerase is complexed with the 'pre-terminal protein' which binds dCTP and then attaches covalently to the ends of the linear genome during priming. In the presence of the virus-encoded DNA-binding protein and two other factors, full-length copies of the adeno genome can be synthesized *in vitro* from purified components.

Recent studies in yeast may advance our knowledge of eukaryotic replicons. Transformation assays have identified a limited subset of DNA segments which allow plasmids to replicate autonomously in yeast. These autonomously replicating sequences have the properties expected of chromosomal 'replicators'. Therefore evidence that replication initiates at these sequences is important and Campbell (CalTech) described a cell-free system which appears to initiate preferentially at autonomously replicating sequences *in vitro*. An additional advantage of yeast is the advanced state of yeast genetics and a permeabilized cell system derived from a thermosensitive cell-cycle mutant (*cdc8*) which can be complemented by fractionated extracts from wild type (Campbell). The transformation assay also led to the isolation of yeast centromere sequences which confer mitotic and meiotic stability on plasmids replicating in yeast. Carbon (University of California, Santa Barbara) reported another effect of the centromere — control of copy number of the endogenous 2μ plasmid of yeast. When a centromere is introduced, the copy number falls from around 50 to only 1 per cell.

Yeast has advantages over higher eukaryotes for the study of replication but Hamlin (University of Virginia Medical School) described a promising approach for studying a replicon in Chinese hamster ovary cells. The gene coding for dihydrofolate reductase becomes amplified in response to methotrexate selection. The degree of amplification

Vive le replicon!

from Ron Laskey and Marcel Mechali

TWENTY years ago Jacob, Brenner and Cuzin^{1,2} proposed a fundamental unit of DNA replication, the replicon. They argued that tight coupling of DNA replication to other cellular events was regulated by the action of a diffusible positive factor, 'the initiator', on a specialized chromosomal region, 'the replicator'; resulting in replication of the entire circular chromosome of a bacterium, plasmid or bacteriophage. The twentieth anniversary of the replicon hypothesis was celebrated by a meeting* which considered its impact on current studies of prokaryotic and eukaryotic DNA replication.

The chromosomal replicator of *Escherichia coli* has been mapped to within a 245 base pair (bp) fragment from the *oriC* region. This is sufficient to direct initiation of replication *in vitro*. Surprisingly, polymerization does not begin in this fragment itself but in the upstream flanking DNA, irrespective of the nucleotide sequence of the flanking region (Hirota, National Institute of Genetics, Japan). Replication subsequently becomes bidirectional after an asymmetric start.

The product of gene *dnaA* behaves as an initiator for the chromosomal replicon of *E. coli* and a cell-free system from *E. coli* requires the *dnaA* protein for initiation at *oriC* (J. Kaguni, Stanford Medical School). Fractionation of this system has produced an intriguing result. As well as several identified components³ and at least two unidentified positive factors, an

additional template-specificity factor is required for initiation at the *E. coli* replication origin. In the absence of this factor the amount of DNA synthesis increases, but synthesis no longer requires the *dnaA* gene product and it is no longer template specific for *oriC*. Apparently the enzymes of replication could initiate elsewhere and the replicon concept has acquired another component which constrains initiation to the site specified by the initiator.

In the initiation of DNA replication two discrete roles for RNA synthesis have been identified. First, DNA chains are usually primed by synthesis of oligoribonucleotide primers⁴. Second, there are several examples of transcriptional activation of DNA replication in which the origin region is transcribed to form a transcript which allows a true priming event subsequently to occur. A particularly good example is phage λ where a pause during transcription through the origin appears to expose the site for priming in a single-stranded form (Hobom, Albert Ludwigs University, Freiburg). Alternatively, in the replication of plasmid Col E1, interaction of the long RNA primer with a complementary transcript from the opposite strand controls plasmid copy number (Tomizawa, NIH). In phage T7, two promoters for T7 RNA polymerase flank the primary replication origin and appear to be involved in replication initiation. They have no open reading frame between them and their deletion inhibits replication from that origin (Richardson, Harvard Medical School). Conversely, deletion of the primary replication origin results in initiation at alternative sites which are also near promoters for T7 RNA polymerase. During elongation nascent T7 DNA chains

* A meeting entitled 'The Replicon, 20 years after' was sponsored by INSERM at Seillac, France, on 19–23 September 1982.

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allows restriction fragments from this gene and its flanking sequences to be recognized in digests of total DNA. Pulse-labelling with thymidine reveals that the characteristic restriction fragments replicate early in the S phase of the cell cycle and that there is a temporal order of fragment labelling. Isolation of these fragments and insertion

into other cells could help in elucidating the nature of the elusive eukaryotic 'replicon'.

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Is polypeptide heterogeneity the key to neurofilament function?

from Rosaleen Calvert

THE most enigmatic component of the cytoskeleton remains the intermediate filament. Microtubules and microfilaments can depolymerize to tubulin and to actin respectively, and the effects of their absence in the cell investigated. In contrast, there are no situations where intermediate filaments depolymerize *in vivo* (though other ultrastructural changes do occur) and no known drugs which have this effect. Nonetheless, by using a variety of approaches we are now beginning to find out what the functions of these filaments may be. One class of intermediate filaments — the neurofilaments — is the subject of this article (see also refs 1,2).

Intermediate filaments were first defined ultrastructurally as filaments intermediate in width between the thick filaments of myosin and thin filaments of actin³. Subsequent purification from different tissues led to the surprising observation that although the proteins actin and tubulin always consisted of one and two polypeptides respectively, there were five classes of intermediate filaments. In the case of the epithelial cytokeratins, they

contain at least nineteen different polypeptides in contrast to, for instance, the fibroblast intermediate filament protein vimentin which is a single gene product. The three remaining intermediate filaments are desmin in muscle, glial fibrillary acidic protein in glia and neurofilaments. Mammalian neurofilaments consist of only three polypeptides of molecular weight about 200,000, 150,000 and 70,000 (ref.4); these will subsequently be referred to as the 200K, 150K and 70K. It has proved fruitful to probe their different roles in neuronal structure and behaviour.

The first indication of heterogeneity in neurofilament structure came from a genetic polymorphism of the 200K in rabbits, in which one variety had a molecular weight about 10,000 larger than the other. Heterozygotes and homozygotes for each allele were observed. Unfortunately, no functional attributes of these animals mirror their molecular differences⁵. There

is further heterogeneity in the isoelectric points of all three polypeptides⁶, but again, the functional significance is not yet clear.

Recently, immunofluorescence has revealed neuronal processes which lack or have very little of the highest-molecular-weight polypeptide — the dendrites of pyramidal cells of the cerebral cortex and hippocampus and certain cell bodies or axons in the brain stem on sections of adult rat⁷ and human⁸ brain, and certain dorsal root ganglion^{9,10} neurones. In developing rat brain the 200K can be first detected with Coomassie brilliant blue stain on a gel three days postnatum, whereas the 150K and 70K can be seen one day prenatum. It could be therefore that the 200K is found only in mature axons¹¹. However neurofilaments are known to be susceptible to an endogenous calcium protease and there is some evidence that this is not evenly distributed, at least within single neurones¹², so it is possible that the 200K is proteolysed during tissue preparation.

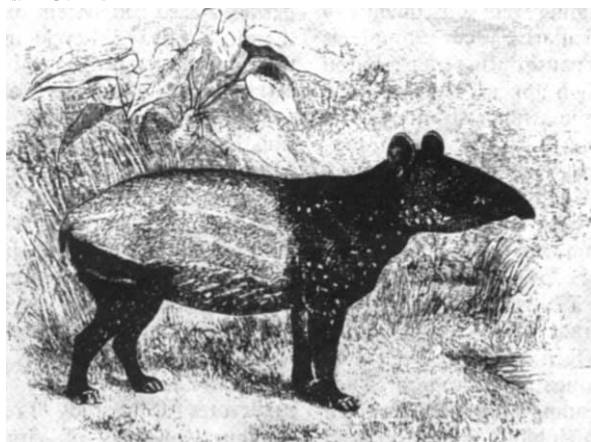
Ten nm filaments can be reconstituted from the 70K alone¹³⁻¹⁵ or with the 200K added, and thin sections show projecting side arms¹³, suggesting that the 200K could be responsible for the bridges between neurofilaments observed in the electron microscope. There is some immunological evidence for this: antibodies specific for the 200K appear to label the cross-links between neurofilaments in electron microscope studies of whole neuronal cytoskeletons¹⁶. Moreover, *in vitro* digestion studies using trypsin have shown that 75 per cent of both the 200K and the 150K can be removed, leaving morphologically intact filaments¹⁷.

It is interesting that in the growing axon, the neurofilament triplet is transported distally at eight times the final rate. Retardation correlates with the increasing quantities of the 200K which appear at this time, and it has been proposed that a concomitant formation of cross-bridges could be the explanation for the change in rate of axoplasmic transport¹⁸. This anterograde transport of neurofilament polypeptides also raises the question of what happens to the filaments or networks of filaments when they reach the axon terminal. Soluble subunits have never been found, so degradation is still considered as the most plausible mechanism for their disposal¹⁹. In support of this mechanism is the observation that a monoclonal antibody^{20,21}, which binds strongly to the 200K and weakly to the 150K, binds to these and only two other polypeptides in neonatal rat brain whereas in the course of further development a whole ladder of antibody-binding polypeptides appears below the 200K on polyacrylamide gels. A possible interpretation of this observation is that gradual cessation of axonal outgrowth necessitates an increase in degradative processes to dispose of neurofilaments accumulating at the axonal terminals. The 200K polypeptide thus emerges with a distinct structural role. Further compari-

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100 years ago

NEW OR RARE ANIMALS IN THE ZOOLOGICAL SOCIETY



THE MALAYAN TAPIR (*Tapirus indicus*). There is no better instance of discontinuous distribution on the world's surface than that of the Tapirs. While Tropical America contains several species of *Tapirus*, and may

be regarded as the *focus* of the genus, a single well-marked species occurs in Tropical Asia. This is the Malayan or Indian Tapir, *Tapirus indicus* (sive *malayanus*) of systematists. From *Nature* 27, 151, 14 December 1882.

son of the situations in which it is present or absent may provide more insight into its function.

Special features of the other two neurofilament polypeptides are scarce. In a study of polypeptide compositions of different sections of the axons in a mouse optic tract, the 150K exhibited microheterogeneity. Two major species were visible along the length of the optic pathway with an extra one appearing distally. The amount of this third polypeptide could be increased by incubation of the intact optic pathway *in vitro* with a calcium-containing buffer, suggesting the presence of a protease which was absent or inactive in the proximal part of the axons¹². This again supports the idea that neurofilaments are degraded as they reach the axon terminal.

The anti-neurofilament monoclonal antibody²⁰ mentioned above also binds to a high-molecular-weight polypeptide present in developing rat brain and absent in other tissues of neonatal rat. Thus by immunological criteria there seems to be another neurofilament polypeptide associated with immature brain²¹. Whether other properties of this polypeptide, such as solubility and secondary structure, will confirm its assignment as an intermediate filament protein remains to be seen.

An interesting selection of rather different neurofilament structures is now

accumulating. Those situations where a change in structure can be seen and correlated with a physiological event offer the best chances of using structural differences to throw light on the elusive neurofilament function. □

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by repeated duplications, but was constrained by the absolute necessity of maintaining the repeating Gly-X-Y structure in the protein.

Another remarkable feature of the evolution of the collagen genes is the way that the amino acid sequence and nucleotide sequence have been conserved around the carbohydrate attachment site in the C-terminal propeptide region in chick α_2 (I) and human α_2 (I) (D. Prockop, Rutgers University). Similar conservation in this region has been observed in the sheep α_2 (I) gene⁷, the chick α_1 (II) gene (B. Olsen, Rutgers University) and the chick α_1 (III) gene. It is easy to think of functional reasons why such conservation occurs at the amino acid level but lack of third-position codon change in this region is striking, implying selection at the nucleotide level for the conservation of the sequence and making the region a fascinating one to explore — it may perhaps function as the attachment site for a regulatory molecule.

Do all collagen genes have the same basic structural features and have a common evolutionary history? From recent studies on the structures of fruitfly^{9,10} and nematode¹¹ collagen genes, the answer to at least the first question is clearly no. The cuticle nematode *C. elegans* contains a large number of collagenase-sensitive cuticle proteins, and the genes for two of these have been isolated (D. Hirsch, University of Colorado). These two genes are not closely linked, are related but not identical and are each about 1,200 bp long. Sequences coding for Gly-X-Y repeats are seen in both of the genes, but none of the exons is 54 bp or variants thereof, or a multiple of 9 bp. In fact, the first gene is divided into only three exons, the second into two. Analysis of a fragment of a *Drosophila* collagen gene (J. Monson, University of California, San Francisco) has revealed two large regions, of 662 and 726 bp, which encode a region of 460 amino acids. The structure of this gene is again quite different from that of the vertebrate interstitial collagen genes. The *Drosophila* collagen protein appears to be closely related to basement membrane type IV collagen (J. Fessler, University of California, Los Angeles) and cross-reacts with antibodies to mouse type IV collagen. The big question now is: what is the structure of the genes for vertebrate basement membrane collagens — are they similar to the interstitial collagen genes or like that of the nematode and *Drosophila*? The answer obviously is crucial to our understanding of the evolution of collagen genes.

Analyses using dot hybridization on genomic human placental DNA (R. Dalgleish, NHLBI, Bethesda) indicate that

Collagen genes

from Paul Tolstoshev and Ellen Solomon

THE collagen genes, a major family of at least fifteen genes found throughout the vertebrate and invertebrate kingdoms, have been much studied, not only for what they can tell us about the molecular biology of evolution but also because they are developmentally regulated, crucial for the structural integrity of most tissues and are implicated in many human mendelian genetic disorders. Reports delivered at a recent meeting* revealed progress in understanding of all these areas.

All collagenous proteins have in common a triple-helical region with a repeating pattern in which every third amino acid is glycine. The interstitial collagens (types I, II and III) form rigid rod-like structures while basement membrane collagen (type IV) forms a different kind of molecule, still with large amounts of triple helix, but with nonhelical interruptions.

A very detailed picture of the gene for the α_2 chain of type I collagen¹⁻³ is now available (H. Boedtker, Harvard University). The most remarkable feature of this gene, apart from its size and that it

contains about 50 introns in 40 kilobases (kb) of DNA, is the unique conservation of the 54 base pair (bp) exon size throughout the α -chain-coding region. There has been much speculation as to the significance of this structure in the evolution of collagen genes^{4,5} and the analysis of other collagen genes has been awaited with great interest. Information is now available for mouse α_1 (I) (ref. 6), chick α_1 (II) (B. Upholt, University of Chicago), chick α_1 (III) (Y. Yamada, NCI, Bethesda), human α_2 (I) (F. Ramirez, Rutgers University) and an α_1 (I)-like gene⁷. All these genes have conserved the basic 54 bp (18 amino acid) exon size in the triple-helical coding region of the gene. The size of this exon and its variations [45 bp (54–9), 108 (54 × 2) and 99 (108–9)] are similar to those found in the chick α_2 (I) collagen gene. The only permissible deletions thus appear to be of size 9 bp — one Gly-X-Y triplet. Comparison of particular exons among the different genes indicates that sizes and position are also strongly conserved. Intron sizes, however, vary enormously, from less than 100 bp to several thousand. In general the data are compatible with the idea that the genes had their origin in an ancestral 54 bp sequence which expanded

* The 'International Workshop on the Structure and Regulation of Connective Tissue Genes', was held at Bethesda, Maryland, on 13–14 September 1982.

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there is only one copy of each of the α_1 (I), α_2 (I) and α_1 (I)-like genes per haploid genome in humans. The genes coding for the two chains of human type I collagen are located on different human chromosomes^{12,13}: α_2 (I) on chromosome 7 and α_1 (I) on chromosome 17 (E. Solomon, ICRF, London; F. Ramirez). The presence of the genes for the different chains of heteropolymer on different chromosomes has many well known precedents, and does not allow prediction of whether other related genes will be clustered near either gene.

One of the most studied systems of collagen gene regulation has been the Rous sarcoma virus (RSV)-transformed chick embryo fibroblast (CEF) where the production of type I collagen is dramatically decreased on transformation, probably by regulation at the transcriptional level^{14,15}. The availability of temperature-sensitive strains of the virus has allowed analysis of both the induction and reversal of the transformed state (M. Sobel, NIDR, Bethesda) and has prompted studies of the transcriptional regulatory signals of the chick α_2 (I) collagen gene. The promoter of this gene has been inserted into a hybrid SV40/pBR322 shuttle vector (B. de Crombrughe, NCI, Bethesda) which makes possible detection of promoter activity by measurement of the activity of the product of the *Escherichia coli* chloramphenicol acetyl transferase gene. Analysis of deletion mutants in the α_2 (I) collagen promoter region of this vector has provided some evidence for regulatory sequences, particularly those potentially capable of forming hairpin structures¹⁶. Neither this approach, nor attempts to correlate methylation of the α_2 collagen gene with its activity¹⁷ (C. McKeon, NCI, Bethesda), have been successful in yielding clues to the precise nature of transcriptional regulatory mechanisms. However, a role for methylation in the reduction of activity of type I procollagen gene expression on transformation of human lung fibroblasts with SV40 has recently been reported¹⁸.

Chick embryo chondroblasts, which normally produce type II collagen, when

transformed with RSV strain show decreased synthesis of type II collagen and decreased levels of type II collagen mRNA (ref. 19 and S. Adams, University of Philadelphia). Surprisingly, type I collagen mRNA begins to accumulate, but is not used by the cells to make type I collagen. This is clearly a regulation at the level of translation. Other examples of translational regulation are seen in the effects of the N-terminal propeptides on collagen synthesis in normal and dermatosporotic sheep fibroblasts (P. Müller, Max Plank Institut, Munich) and in the regulation of collagen gene expression in fetal sheep skin development²⁰ and human lung fibroblasts²¹. Clearly, continued analysis of these and other systems using cloned probes will reveal the exact details of the variety of mechanisms cells use to modulate their collagen production.

A variety of human genetic disorders may be due to a structural defect of collagen. These include Osteogenesis imperfecta, several of the Ehlers-Danlos syndromes and the Marfan syndrome. These disorders, while normally inherited in simple mendelian fashion, vary greatly in the severity of their clinical symptoms. Biosynthetic studies of collagen production in cultured fibroblasts from affected individuals show abnormal secretion of collagen in all the three types of disease (P. Byers, University of Washington). The abnormality may be due to underproduction or lack of one constituent α chain, an abnormally long or short chain causing incorrect assembly of the precursor, or inefficient cleavage of the terminal peptides. The wide variability of symptoms has long caused difficulties of classifications of heritable disorders of connective tissue and of meaningful genetic counselling. Classification using biochemical methods is being attempted (D. Rowe, University of Connecticut), but DNA analysis is obviously of prime importance in the understanding of these disorders. Such analyses for two individual cases were reported. A fibroblast culture from an affected individual with the severe perinatal lethal form of OI type II demonstrated both shorter mRNA molecules for α_1 (I) chains and a deletion in the α_1 (I) chain gene in the DNA (M.L. Chu, Rutgers University). The second case is another, less severe form of Osteogenesis imperfecta, where, remarkably, no α_2 (I) collagen chains are found at all in the skin²². While this case is obviously also a prime suspect for a deletion, in fact both pro- α_1 (I) and pro- α_2 (I) mRNAs are present in normal amounts in fibroblasts (D. Prockop, Rutgers University). An intriguing study was mentioned (P. Byers; J. Phillips, John Hopkins University) of a large Osteogenesis imperfecta family in which the disease segregates completely with a restriction site polymorphism near a growth hormone gene on chromosome 17. The suggestion is one of linkage of the disease gene (possibly the α_1 (I) chain gene)

with the polymorphism, and is the first instance of this kind of analysis for a connective tissue disease. The analysis of more cases of the genetic connective tissue disorders is underway with the probes that are available, and the results will be of great basic as well as clinical interest. □

Onco Gen

from Peter Newmark

A paper on page 607 of this issue implicates the cellular gene known as *c-mos* in malignancies of antibody-producing cells or their precursors and illustrates a novel way of activation of an oncogene.

The *c-mos* gene is so called because it is the cellular homologue (and presumably the precursor) of the oncogene carried by the Moloney murine sarcoma virus. Whereas the viral gene is oncogenic, *c-mos* is not unless activated. In 1980 Blair *et al.* (*Science* 212, 941) demonstrated activation of *c-mos* by the attachment to it of the long terminal repeat sequences of the Moloney murine sarcoma virus, probably on account of increased expression of *c-mos* under the influence of the long terminal repeats.

Rechavi *et al.* now demonstrate a completely different mechanism of activation of *c-mos* by — strange though it may seem — the replacement of a sizeable chunk of the gene by extraneous DNA. The mechanism is all the more important because it is not the result of an artificial construct but has been discovered in a chemically induced mouse myeloma.

The evidence comes from a comparison of the sequences of the normal *c-mos* gene and an abnormal version in the myeloma. The abnormal version, which was able to transform NIH 3T3 fibroblasts — the standard assay for an oncogene — had lost over 500 nucleotides from one end, including the information for the first 88 amino acids of the *c-mos* protein.

Intriguingly, a 159-nucleotide block of DNA immediately adjacent to what is left of *c-mos* has the hallmarks of a transposable genetic element, suggesting that a transposition has displaced some *c-mos* nucleotides and activated what is left.

The mechanism of the presumed activation is as much, if not more, of a mystery than in the case of the point mutation that seems to have produced the human bladder carcinoma oncogene (*Nature* 300, 143 & 149).

In a note added in proof, Rechavi *et al.* claim that they have also detected a rearranged *c-mos* gene in a Burkitt's lymphoma. It is not yet known if the rearrangement involves a transposition but it does seem to be the consequence of a translocation from chromosome 8 to 14, bringing the *c-mos* gene into the proximity of the variable-region heavy-chain immunoglobulin gene in a fashion similar to that already reported for the *c-myc* gene (see *Nature* 300, 403).

Peter Newmark is deputy editor of *Nature*.

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REVIEW ARTICLE

The T lymphocyte antigen receptor—paradigm lost

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It has been dogma that the antigen receptors on T lymphocytes must have recognition sites that are essentially the same as those of antibodies and that the receptors would be found if structures related to immunoglobulin V-domains could be identified on T lymphocytes. In a critical assessment we conclude that there is no sound basis for these views.

ANTIBODIES are the only well-characterized molecules that are known to bind foreign antigens in the immune response. An enormous range of antibodies can be produced with their different specificities being due to different amino acids in the combining site.

Lymphocytes are the only cells known to be capable of specific recognition of foreign antigen. This recognition results in the stimulation of antibody secretion and the activation of cells responsible for cell-mediated immunity. B lymphocytes (bursal or bone marrow-derived) give rise to antibody-secreting cells while T lymphocytes (thymus-derived) are responsible for the cell-mediated functions which include cytotoxicity and the control of other immune responses. Helper T cells provide a stimulus which is usually essential for the differentiation of B cells and cytotoxic T cells. Suppressor T cells turn off immune responses.

The antigen receptor on B lymphocytes is antibody and it has long been argued that the antigen receptor of T lymphocytes should at least use the antibody gene pool to encode its combining site regions. This point of view derives from the argument that it is unlikely that the problem of recognizing antigen should have been solved twice in evolution. However, we conclude here that there is no good evidence for the expression of antibody genes in T cells and many data suggest the contrary. Furthermore the discovery of molecules related to immunoglobulin (Ig) does not necessarily lead to the identification of immune receptor molecules.

Receptors on B lymphocytes

Figure 1a shows a model for a receptor IgM at a cell surface^{1,2} and defines the terms used to describe antibody structure. The

Fig. 1 The structure of receptor antibody. *a* Shows IgM antibody at a cell surface. The structure consists of two identical heavy (H) and two identical light (L) chains held together by disulphide bonds (SS symbols between the chains). There are two identical antigen combining sites, each formed from the N-terminal parts of one H and one L chain. The H and L chains can be divided into domains which show sequence homology (illustrated by circles) including a conserved intra-chain disulphide bond (S symbols). The N-terminal domains are called V domains (or regions) because their amino acid sequence varies to give different antibody combining specificities. The other domains are called C domains because their sequence is constant for any one L-chain type or H-chain class. The C domains for any one chain are collectively referred to as the C region. The symbols † show attachment points for N-linked carbohydrate structures. *b* and *c* show the folding pattern for the polypeptide of V and C domains respectively. In both sorts of domain anti-parallel β -strands form two β -sheets that are held together by hydrophobic interactions and the conserved disulphide bond. The bar indicates the conserved intra-chain disulphide bond and the letters, the β -strand sequences labelled sequentially along the sequence. The V domain has an extra loop of sequence in the middle and the β -strands in this are indicated by C' and C''. The loops of sequence in V domains which are believed to form the combining site show great variation in amino acid sequence between different V domains and are indicated with the label: hypervariable regions (the domains are redrawn from ref. 3).

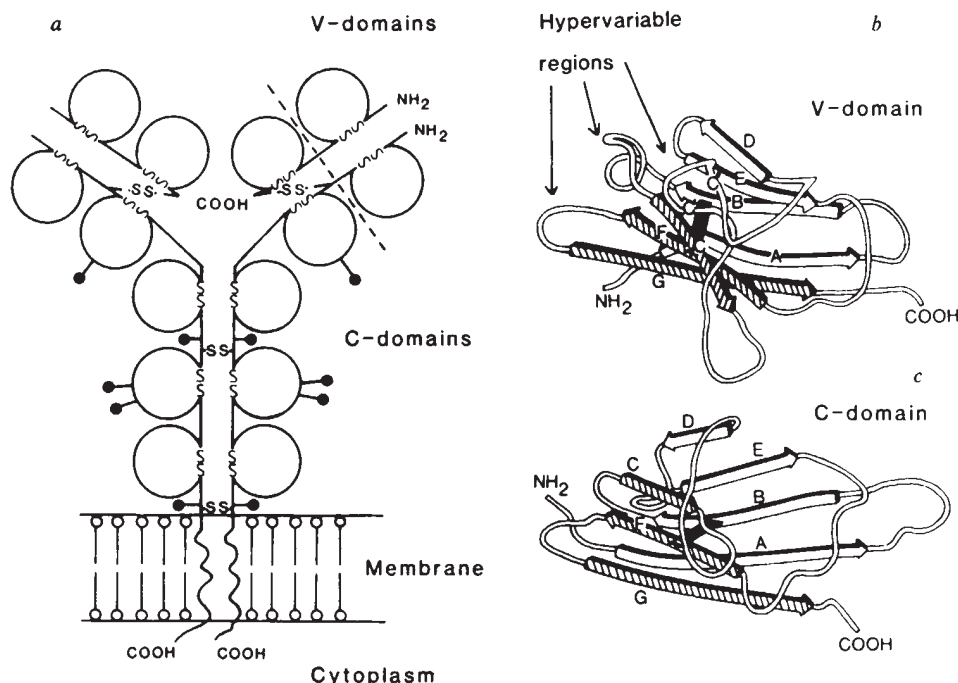


Table 1 Chromosomal assignments of genes for immunoglobulin and immunoglobulin related molecules in the mouse

Molecules		Chromosome (ref.)
Immunoglobulins		
Lambda:	V _λ C _λ	16 (152)
Kappa:	V _κ C _κ	6 (153)
H-chains:	V _H C _H μγ ₃ γ ₁ γ _{2b} γ _{2a} εα	12 (154)
Major histocompatibility complex antigens		
Class I:	H chains	17 (19)
Qa:	H chain	17 (155)
Class II:	α and β chains	17 (19)
	β ₂ -microglobulin	2 (156)
Differentiation antigen		
Thy-1		9 (19)
T receptors		
		?

antibody heavy (H) and light (L) chains can be subdivided into domains of about 110 amino acids which show sequence and structural homology^{3,4} (Fig. 1b, c) but have quite different functions. The variable (V)-domains form the antigen recognition site while the constant (C)-domains of the H-chains function as determinants to be recognized by molecules of the immune effector systems (for example the complement cascade) which are triggered after antibody has bound antigen^{5,6}.

The genes for the V and C regions of any one immunoglobulin chain are linked but separated by a considerable distance on the chromosome. There are multiple V-domain genes but only one C-region gene for each chain. In ontogeny of B lymphocytes one V_L and one V_H gene is spliced to a new position closer to their respective C-region genes, committing the cell to one combining site^{7,8}. In further differentiation genetic translocations can lead to expression of different C regions in the H chain⁹ but the expressed V_H and L chains remain the same unless somatic mutation occurs.

The commitment of B cells to one combining site specificity can be demonstrated at the cellular level. Antibody at the B-cell surface can bind soluble or particulate antigen^{10,11} and the antigen-binding cells for a typical antigen are usually found at a frequency of about 10⁻⁴. These cells can be separated from other lymphocytes in various ways and have been shown to include the precursors for antibody-secreting cells of the appropriate specificity¹²⁻¹⁴.

Antigen recognition by T lymphocytes

An individual T lymphocyte must have a limited set of endogenous antigen receptors since the immune functions carried out by T-cell clones are antigen specific¹⁵. In exhibiting this clonal specificity T lymphocytes are like B lymphocytes; another similarity is that T cells can distinguish a wide range of different antigenic structures¹⁶⁻¹⁸. However, T- and B-cell responses often do not coincide. For example a large number of minor transplantation antigens have been defined by T-cell responses but antibodies to them have not been produced¹⁹. Also some histocompatibility antigen mutants provoke T-cell responses but no antibody formation²⁰. In other cases antibodies distinguish different antigens but T cells show cross-reaction¹⁶. This is commonly seen in immune responses to viral antigens²¹.

One major difference between B cells and helper or cytotoxic T cells is that these T cells cannot directly bind soluble or particulate antigen that has not been processed by macrophages. Past claims to have identified antigen-binding T lymphocytes have been, at best, controversial. Acquired antibody can easily lead to false positives^{22,23} as can the fact that the commonly used antigen TIGAL may bind to small numbers of T lymphocytes by cross-reaction with Fc receptors²⁴. Acquired antibody is not a problem in studies with bursectomized chickens

which have functional T lymphocytes but no B lymphocyte serum antibody. Antigen-binding T cells cannot be detected in these animals^{22,24-26}. Similarly in mice there is no evidence for direct binding of antigen by helper or cytotoxic T cells if the criterion used is the enrichment or depletion of functionally active T cells on the basis of antigen binding^{17,27-31}.

The situation is different for suppressor T lymphocytes, which do show antigen binding^{28,32,33} and also secrete factors which bind antigen³⁴. In these properties suppressor T cells are similar to B lymphocytes and this analogy is supported by the finding that suppressor cells distinguish native and denatured antigen (as do B cells) whilst helper T cells do not make this distinction²⁸.

The difficulty in finding antigen-binding T cells became explicable when it was shown that T lymphocyte helper and cytotoxic responses require simultaneous recognition of foreign antigen along with self-histocompatibility antigen at the surface of an antigen-presenting cell^{35,36}. Functional T lymphocytes will bind to a monolayer of macrophages which have been loaded with antigen³⁷. Cytotoxic T lymphocytes usually recognize antigen along with class I histocompatibility antigens whilst recognition by helper T lymphocytes usually involves co-recognition of class II antigens³⁶.

The differences in antigen recognition by T and B cells suggest major differences in their receptors but do not rule out the possibility that T-cell receptors utilize at least a part of the antibody structure. Initially it seemed possible that T receptors were very similar to antibodies but that they possessed a different H chain. This is the IgX concept³⁸ (Fig. 2a) and is based on the fact that the different classes of antibodies are specialized for different effector functions. Subsequently the IgX model was modified to that shown in Fig. 2b in which the only immunoglobulin products shared with B cells are the V_H domains³⁹⁻⁴¹.

The search for T lymphocyte immunoglobulin

Antibodies have been widely used to search for immunoglobulin on T cells. Antibodies combine with a relatively small area of an antigen and antibodies against immunoglobulin are likely to recognize sequences at the exposed corners or surfaces of the immunoglobulin domains (Fig. 1b, c). By immunization between species it is simple to produce antibodies reacting with C regions of L chains and H chains and it is also possible to prepare anti-V-region antibodies which react with conserved determinants shared by many antibodies. In fact, anti-V antibodies are common components in sera raised against whole immunoglobulin^{42,43}. Other anti-V antibodies can be against the variable parts of V domains (anti-idiotypes, see below) and these react with a minor sub-set of total immunoglobulin^{44,45}.

If the IgX concept (Fig. 2a) is right, anti-immunoglobulin antibodies that include anti-L-chain antibodies should label T lymphocytes. The failure to detect such labelling^{25,46}, by any but the most sensitive methods, must be one of the most repeated observations in immunology. In experiments using the fluorescence activated cell sorter the labelling of T lymphocytes with anti-immunoglobulin antibody is invariably indistinguishable from background (for example, ref. 47). If the T cells bound 10% as much antibody as B cells, this would be detected and it is thus clear that T lymphocytes do not routinely label with anti-immunoglobulin reagents at 30% of the B lymphocyte level as has been claimed⁴⁸. However, using autoradiography specific labelling of T lymphocytes with anti-immunoglobulin antibody can be detected at about 100-1,000 molecules per cell compared with about 10⁵ molecules for B lymphocytes^{49,50}. The origin of the immunoglobulin detected has been studied in the rat using anti-L-chain allotype antibodies⁵¹ and in the chicken using normal and bursectomized birds⁵². In both cases it was concluded that the small amount of immunoglobulin detected was acquired from exogenous sources. L chain was also searched for in extracts from T lymphocytes of the bursectomized chicken and < 100 molecules per cell was detected⁵³.

To our knowledge there are no experiments in which the serological determinants of any immunoglobulin constant region have been found on T lymphocytes and been shown to be of endogenous origin (see controversial positives, below).

Given the above results and data from experiments with anti-idiotypic antibodies^{39,40} (see below), a model for the T-cell receptor as shown in Fig. 2b became accepted⁴¹. This is characterized by the expression of antibody V_H regions which should be detectable with anti-V-region antibodies specific for conserved determinants. Suitable antibodies can be prepared against the rabbit 'a' allotypes which are polymorphic determinants common to all immunoglobulin classes⁵⁴ and expressed on isolated H chain and V_H fragments⁵⁵. Using these antibodies, attempts have been made to find molecules with V_H but not L-chain determinants by labelling of rabbit lymphocytes and analysis of cell extracts. In seven independent studies (refs 56–62) no evidence was found for these molecules, although expression at a few thousand molecules per cell may have gone undetected. Stimulation *in vivo* with antigen⁶², or *in vitro* with mitogens or allogeneic cells⁶³ did not alter this negative result.

One report describes antigen-binding rabbit lymphocytes which express V_H but not L-chain determinants⁶⁴ and another, antigen binding molecules with these characteristics⁶⁵. These results are difficult to evaluate without further analysis and an understanding of the nature of antigen binding T cells and factors (see above).

Other antibodies which react with V domains of a large fraction of immunoglobulin have also been produced. Two groups have used chicken or mammalian antibodies against rabbit V_H determinants distinct from the 'a' allotypes including antibodies that reacted only with free V_H or H chain^{60,63}. V_H unassociated with classical immunoglobulin was not detected. Others have prepared antibodies against mouse V domains. A monoclonal anti-mouse V_H antibody did not react with T cells⁶⁶ and rabbit anti-mouse F_V (includes V_H and V_L) did not label thymocytes or T cells⁶⁷. In contrast, similar rabbit antibodies against mouse V domains have been reported to inhibit antigen binding by T cells⁶⁸, but to show no binding to T-cell clones¹⁷. Again the positive effect is difficult to interpret due to the uncertainties about antigen-binding T cells. A rabbit anti-human V_H antibody was reported to show inconsistent labelling of human T cells⁶⁹.

To summarize, the evidence for V domains on T cells from experiments with widely reacting anti-V antibodies is unconvincing (particularly since there are a variety of ways that false positives can occur, see below).

Experiments with nucleic acid probes

Nucleic acid probes provide alternative reagents for searching for the expression of antibody genes in T cells. The probes can

be used to search for mRNA in the T-cell cytoplasm or to examine the DNA of T cells to see if V-region genes have been moved in the manner that occurs in the commitment of B lymphocytes. In preparing probes DNA is copied (cDNA) from purified immunoglobulin mRNA and then cloned in plasmids to ensure the purity of the probe. Problems with the purity of T cells remain although B-cell contamination is excluded if T-cell clones or lines are studied.

Using cloned cDNA probes L-chain mRNA has not been detected in any T-cell lines^{70,71}. Small amounts of κ mRNA were found in thymus^{71–73} cell extracts but are likely to have come from plasma cells⁷². In a study using uncloned κ cDNA a reaction was detected in most thymus cells by *in situ* hybridization⁷⁴. This result could be due to minor contaminants in the probe. RNA for the C region (C μ) of IgM H chain has been detected in a proportion of T-cell lines^{70,75} and thymocytes^{72,73}. This C μ RNA is not likely to be B-cell-derived⁷². However, similar mRNA was also found in some myeloid cell lines⁷⁰ and in the T-cell lines no evidence for synthesis of C μ polypeptide was found in a rigorous study⁷⁶. The mRNA detected did not appear to contain V_H sequences⁷⁷ and it seems to be an aberrant product made in a variety of early haematopoietic cells. V_H mRNA was not detected in cloned T cells or T hybridomas or functional T-cell lines in a study where four cloned V_H probes were used in conditions that allowed detection of a high proportion of all V_H mRNA in B cells⁷⁸.

Analysis of T-cell DNA showed no rearrangement of L-chain genes as occurs in B cells before immunoglobulin expression^{71,79,80}. Also some studies found no rearrangement of H-chain genes^{71,80,81}. In other cases, H-chain genes of some, but not all, clones or cell lines showed some rearrangement^{75,79,82,83}. In B cells the construction of a functional H-chain gene involves splicing of a V_H segment, which codes for most of the V-region sequence, to a diversity (D) segment and a joining (J) segment⁸. In T-cell clones which showed V_H rearrangement splicing of the D and J segments occurred but rearrangement to produce a functional V_H gene was ruled out in detailed studies on two cytolytic T-cell lines⁸³.

At present, therefore, nucleic acid probes have not clearly aided the problem of identifying the T-cell receptor.

Controversial results not involving idiotypic determinants

The above data indicate that antibody gene products are absent from T cells. Yet, a number of reports claiming identification of T cell immunoglobulin or of cross-reacting molecules of relevance to the T receptor have to be considered.

Anti-immunoglobulin antibodies raised between species widely separated in phylogeny can give heavy labelling of thymocytes or T lymphocytes^{84–87}. However, the reaction of rabbit antibodies against frog^{88,89} or trout⁹⁰ immunoglobulin with thymocytes was shown to be due to a sub-fraction of antibody reacting with carbohydrate. A mouse monoclonal antibody against frog IgM did not label frog thymocytes⁹¹. The labelling of mouse thymocytes with chicken anti-mouse immunoglobulin antibodies was also due to anti-carbohydrate antibody⁹². This was of particular interest since the antibodies analysed were amongst those used by Szenberg *et al.* in their studies⁸⁷ claiming detection of mouse T-cell immunoglobulin.

Other claims for positive results have been made when immunoglobulin was detected by immunoprecipitation in extracts from T lymphocytes or thymocytes. Cone and Marchalonis have consistently reported positive results in studies^{93–95} using cells that have been surface-labelled with ¹²⁵I. A number of other groups have not supported these findings^{96–98}. Cone attributed success to the methods used to extract membrane proteins. Urea-acetic acid was initially favoured⁹³ and subsequently very low levels of detergent were recommended⁹⁴. However, quantitative studies showed urea-

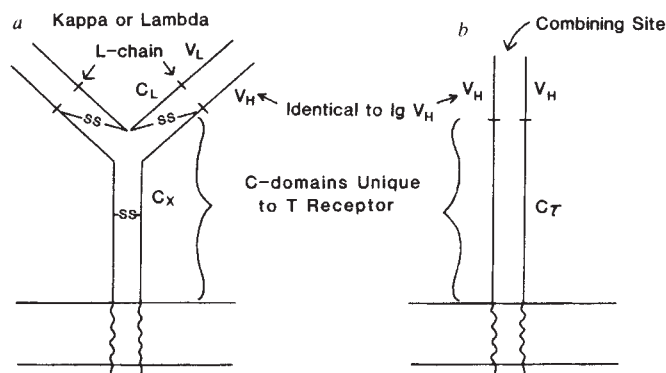


Fig. 2 Models for immunoglobulin related T lymphocyte antigen receptors. In both models the polypeptides differ from the homologous regions of B-cell antibody only in C_x or C_T regions which are the products of postulated genes for constant regions expressed only in T lymphocytes (a based on ref. 38, b on refs 39–41).

acetic acid gave very poor recovery of B-cell immunoglobulin^{53,99,100} and very low levels of detergent might release membrane proteins as aggregates¹⁰¹. This could give misleading immunoprecipitations.

In biosynthetic labelling studies immunoglobulin has been detected in extracts from thymus cells^{102,103}. However, the amount of thymus-derived immunoglobulin varies greatly between animals^{53,100} and cannot be detected if thymus cells are treated to remove B cells before preparing extracts^{98,104}. Relatively large numbers of plasma cells can be found in the thymus¹⁰⁵ and these are likely to be the source of thymus immunoglobulin. Removing all contaminating B cells is difficult since not all plasma cell precursors are readily removed by procedures commonly used to deplete Ig⁺ cells from a lymphocyte mixture¹⁰⁶.

Finally, a general problem is the possibility of cross-reactions with the many immunoglobulin related membrane molecules (Table 1) which are not relevant to the T receptor. Anti- κ chain antibodies which react with β_2 -microglobulin have been described^{107,108}.

Idiotypic determinants on T lymphocytes

Idiotypic antigenic determinants are displayed in the V regions of antibodies and may often be located in the loops of amino acid sequence making up the combining site^{44,45}. In many—although not all^{109,110}—cases they correlate with the combining site specificity of the idiotype-positive antibody. However, anti-idiotype antibodies do not necessarily react only with idiotype-positive antibodies. Anti-idiotype antibodies raised against antibodies specific for physiological effector molecules (for example, insulin) have been shown to recognize the physiological receptors for the molecule concerned^{111–114} even though there is no evidence that these receptors are immunoglobulin

related. Anti-idiotype antibodies labelled early haematopoietic cells which were immunoglobulin negative¹¹⁵ and in another case an antibody to Thy-1 antigen reacted with the TEPC-15 idiotype¹¹⁶. Thy-1 is homologous to V domains¹¹⁷ and its cross-reaction with TEPC-15 myeloma may be a chance event between two related molecules. Finally, a molecule was identified from a T-cell hybridoma which bound azobenzene-arsenate hapten and was recognized by antibodies to an idiotype associated with anti-hapten antibodies. However this was of no relevance to T receptors as the molecule has now been found in the cytoplasm of many cells¹¹⁸.

Given the above, it is clear that a reaction between an anti-idiotype antibody and a cell or cell product does not provide a definitive assay for the products of antibody V genes. Yet this interpretation has consistently been applied to studies which show that anti-idiotype antibodies can affect helper or suppressor T-cell functions *in vivo* or *in vitro* or react with suppressor factors in ill-defined cell extracts.

In a variety of systems anti-idiotype antibodies can be raised against a related set of antibodies which predominate in the response to a given antigen in a particular mouse strain¹¹⁹. The expression of the idiotypes in the antibody response is often linked to the immunoglobulin H-chain constant region allotypes. The anti-idiotype antibodies can stimulate helper or suppressor T lymphocytes specific for the given antigen^{40,119–122}. These effects are only seen in the mouse strains which carry the immunoglobulin heavy chain haplotypes which correlate with the appearance of the idiotype in the antibody responses^{40,121,122}. One interpretation of these data is that the T cells express the idiotype-positive V domains⁴⁰. However, an alternative is that the idiotype determinants are expressed in non-immunoglobulin framework sequences and that T-cell clones expressing these determinants are selected by regulatory events dependent on the expression of the antibody idiotypes¹²³. Until detailed molecular data on the T cell products are available the phenomena cannot be interpreted.

In other experiments suppressor factors in cell extracts have been shown to bind specifically to anti-idiotype antibody columns³⁴. Again no molecular interpretation is possible until the factors are characterized in detail. It may not be too long before one has a detailed analysis of suppressor factor specific for keyhole limpet haemocyanin, because the translation from mRNA of products with this activity has recently been described¹²⁴. This should lead to isolation of the suppressor factor genes.

Experiments on idiotypes of T-cell receptors for the major transplantation antigens appeared to hold hopes for molecular analysis. Binz and Wigzell prepared anti-idiotype antibodies against these receptors which labelled the functionally relevant cells and cross-reacted with antibodies of the same specificity³⁹. They reported purification of the receptors and prepared rabbit and chicken antibodies against them^{125,126}. This was all completed by 1976, yet there has been no further progress and no convincing repeats by other laboratories¹²⁷. It is surprising that the pure receptor has not been used to raise monoclonal antibodies. It seems likely that the situation is not as clear as was at first thought and that difficulties of interpretation apply even though data which appeared decisive were reported.

In our view the idiotype data have been consistently over-interpreted and this criticism can also be made of recent data which claim the identification of allotypic determinants on the constant region of T receptors. Conventional¹²⁸ and monoclonal^{129,130} alloantibodies have been prepared which react with determinants linked to immunoglobulin H-chain genes and it is claimed that they react with T receptors since the antibodies inhibit a reaction between T cells and antibody idiotypes¹²⁸ or react with T suppressor factors¹³⁰. How reliable these systems are for assaying T receptors remains to be seen but it is disappointing that only cytotoxicity analysis, which is notorious for possible artefacts, has so far been used to determine with which cells the antibodies react¹²⁹. Quantitative binding studies using the fluorescence-activated cell sorter have not been carried out

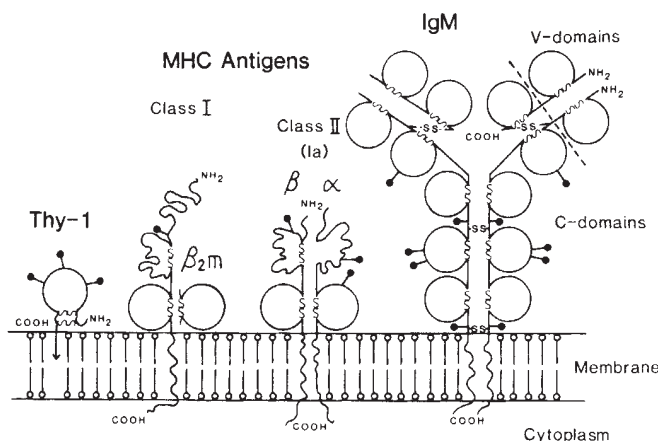


Fig. 3 Immunoglobulin-related molecules found at lymphoid cell surfaces. The diagrams are drawn roughly to scale and represent the polypeptide chains of: Thy-1 differentiation antigen; the major histocompatibility complex (MHC) class I and class II antigens; and IgM. The class I and class II antigens show homology with immunoglobulin C domains. Thy-1 shows homology with V domains. The circles represent immunoglobulin domains and their homologous regions in the other molecules with intra-chain disulphide bonds shown by S symbols. N-linked carbohydrate structures are shown by NH₂. In the MHC antigens the polymorphic determinants are found mainly in the heavy chains of class I antigens and the β chains of class II antigens. It is highly probable that the class I structure applies to Qa and Tl¹⁴⁵ differentiation antigens as well as to the class I histocompatibility antigens. Qa and Tl have β_2 -microglobulin (β_2 -m) in their structure and a gene which maps to the Qa region and may code for a Qa antigen has been sequenced¹⁴⁶ and shown to have an immunoglobulin-like segment as shown for the large chain of class I antigen. The diagrams are based on: Thy-1, refs 117, 131; MHC antigens, class I, ref. 147; class II, refs 36, 148–150; IgM, refs 1, 2. (Reprinted from ref. 151 with permission of the Biochemical Society.)

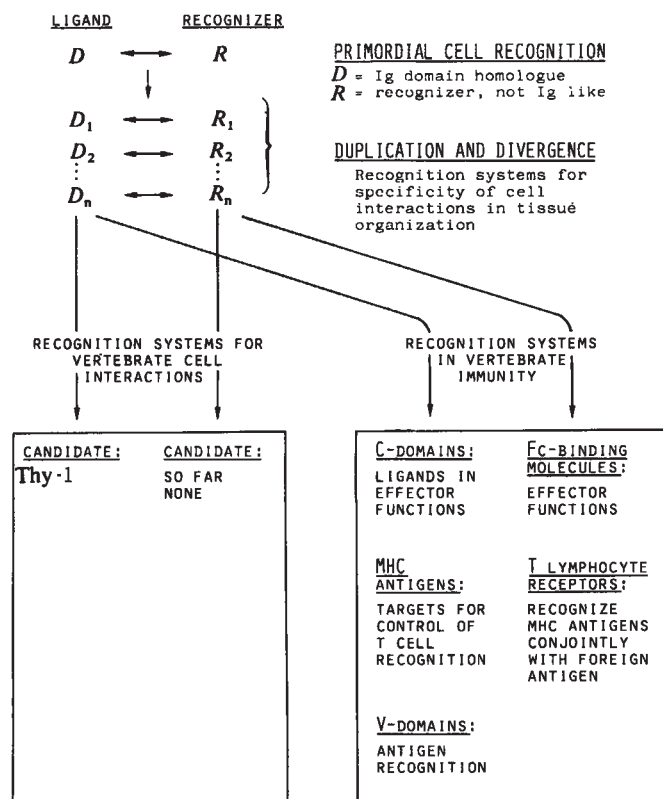


Fig. 4 Possible evolution of recognition molecules for cell interactions and immunity.

and it seems premature to claim that the determinants are part of the T receptor constant regions.

Immunoglobulin-related molecules at the cell surface

A surprising number of cell-surface molecules are now known to have segments homologous with immunoglobulin domains. These are illustrated in Fig. 3 and listed in Table 1. It is remarkable that the list includes all the molecules found on lymphoid cells with sufficient known sequence to allow analysis of homologies. It seems likely that more immunoglobulin related membrane molecules will be discovered.

Experiments looking for serological cross-reactions between antibodies and T-cell receptors imply an assumption that a relationship with immunoglobulin will be diagnostic for molecules concerned with immune recognition. The homologies with histocompatibility antigens may be considered consistent with this since histocompatibility antigens are involved in the presentation of antigen to T lymphocytes. However, Thy-1 antigen cannot be thought of as having a unique role in immune phenomena since in different species its expression is conserved in neuronal cells and fibroblasts but not in lymphoid tissues¹³¹. Thy-1 is homologous with V domains¹¹⁷ and this emphasizes the point that a relationship with immunoglobulin cannot in itself lead to the identification of an antigen receptor.

The genes for the immunoglobulin-related molecules map to six different chromosomes in the mouse (Table 1) and there is no reason why the T-cell antigen receptor genes should be linked to any of these. On the contrary the data suggest they might be quite independently located since there is no linkage between the immunoglobulin genes for H chains and κ and λ L chains.

Alternative possibilities

Given Fig. 1 it seems obvious to say that the T-cell receptor should be related to immunoglobulin as 'everything' else is.

Yet this may not be so, and in Fig. 4 we present a proposal for the independent evolution of antibodies and T-cell receptors from the two arms of a primitive recognition system. We suggest that the role of the primordial immunoglobulin domain was to function as a ligand for triggering cell-cell interactions by binding to a recognizer, which was not immunoglobulin-like, on other cells. The Thy-1 glycoprotein may be a contemporary molecule with such a role^{132,133}. In evolution, genes for both the primordial domain and its recognizer might have duplicated and diverged to produce a set of molecules for determining the specificity of cell interactions in tissue formation. This system may have given rise to antibodies and histocompatibility antigens at the time of vertebrate evolution. The role of the immunoglobulin Fc domains in triggering effector functions and of the histocompatibility antigens in controlling T-cell recognition would be directly analogous to the triggering role of the primitive set of immunoglobulin-related structures. The Fc-binding molecules of the immune effector systems may have evolved from the recognizer arm of the primitive system, and this may have also given rise to the T-cell receptors.

It could be argued that a distinction between ligand and recognizer in systems like this is semantic and also that both arms of the postulated system could be immunoglobulin-related. Amino acid sequences of the various Fc-binding proteins will show whether or not these constitute an independent family of molecules. Thus far the only relevant protein which has been sequenced in C1q and this shows no homology with immunoglobulin¹³⁴.

What next?

We conclude that there is now no good paradigm for thinking about T lymphocyte receptors in molecular terms. Different T-cell clones have different recognition specificities so T receptors must exist but their interaction with antigens is unlike that seen between antibodies and antigens. Somehow there is co-recognition of histocompatibility antigens along with foreign antigen on a presenting cell. The T-cell receptors are unlikely to incorporate any of the gene products seen in antibodies but they may be immunoglobulin-related. However, this does not help greatly in searching for T-cell receptors since many surface molecules are immunoglobulin related.

T-cell receptors can now be sought at either the protein or nucleic acid level. Suppressor T-cell factors are being purified^{124,135-137} and their structure will be of interest since they are the only known T-cell products which seem able to interact directly with antigen. However, their combining sites may be quite different from those of helper and cytotoxic T cells which show co-recognition of self-histocompatibility antigen and foreign antigen. Another approach is to search amongst differentiation antigens recognized by monoclonal antibodies for molecules which are candidates for T receptors. A number of antibodies which inhibit or stimulate T lymphocyte responses have been described¹³⁸⁻¹⁴³. The molecules they recognize may be receptors for foreign antigen or self-histocompatibility antigen or other regulators of T-cell functions. A similar but possibly more direct approach is to look for monoclonal antibodies which distinguish between T-cell clones and thus perhaps recognize idiotypes of the receptors¹⁴⁴. At the nucleic acid level characterization of genes which cross-react with immunoglobulin probes may yet be productive and T-cell-specific molecules can be sought at the level of mRNA. In all these approaches the evidence for relevance to T-cell receptors will be weak until sequence data are available. Only then will it be feasible to try and detect in T-cell clones with different recognition specificities molecules of varying sequences in a constant framework. If such molecules were T-cell specific and if antibodies to them inhibited T-cell functions at the level of primary recognition of antigen, we would be confident that the T lymphocyte antigen receptor had, at last, been identified.

We thank our colleagues for their criticisms and Mrs Christine Scott for assistance with the manuscript.

Note added in proof: Mouse monoclonal antibodies specific for common determinants of human V_H labelled a high proportion of B but not T lymphocytes¹⁵⁷. A molecule which may be mistaken for T-cell immunoglobulin is gp70 of leukaemia

viruses, which is immunoprecipitated from extracts of mouse lymphoma cells by chicken anti-mouse immunoglobulin antibody¹⁵⁸.

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ARTICLES

DSDP Hole 504B, the first reference section over 1 km through Layer 2 of the oceanic crust

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Hole 504B provides a reference section for correlation of in situ petrological and geophysical studies with regional crustal models based on dredged samples and remote surveys; and, at least for the medium-spreading-rate Costa Rica Rift, Hole 504B confirms that the main features of the upper part of an idealized ophiolite sequence occurs in the oceanic crust.

OUR knowledge of the nature of the ocean crust is drawn from three main sources: (1) studies of ophiolite complexes, (2) surveys using remote geophysical tools such as marine seismic reflection and refraction, magnetics, gravity, and heat flow, and (3) petrological and physical measurements on dredged and cored samples. Pioneers^{1,2} recognized early-on that the various rocks dredged from the Mid-Atlantic Ridge were equivalent to the different components of an ophiolite sequence. Several models of the ocean crust have been proposed since which attempted to integrate the various data, but these were usually based on an ideal ophiolite lithostratigraphy (see, for example, refs 3–6). A typical ophiolite complex starts with a pelagic sediment cover resting on effusive pillow basalts, which grade downward into a basaltic dyke complex. This then grades into a plutonic sequence of gabbros and ultramafic cumulates. The lowermost member of the ophiolite sequence is made up of tectonized and metamorphosed ultramafics (ref. 7 and refs therein).

In seismic models of the oceanic crust^{8–10}, fresh or 'weathered' basaltic pillows and flows (Layer 2A) rest under pelagic sediments (Layer 1) and on metamorphosed pillows (Layer 2B), which in turn rest on metamorphosed intrusive dykes (Layer 2C). Originally called the 'volcanic layer'², Layer 2 is on average 2.5 km thick, but its thickness is extremely variable. On the other hand, the underlying 'oceanic layer' (Layer 3) is remarkably uniform in thickness, averaging ~4.5 km, and is thought to be made up of gabbros and more or less serpentinized ultramafics.

The total, seismically-determined thickness of the oceanic crust generally ranges from 5 to 7 km, whereas the total thicknesses of most ophiolite complexes do not exceed 4 km (ref. 7). This, coupled with chemical evidence, suggests that various ophiolites probably correspond to different types of tectonic environments. Thus, one is led to question the validity of using ophiolites as models of ocean crust. Another discrepancy between the ophiolite model and ocean crust is the rarity of dredged and drilled oceanic rocks which can be ascribed to a dyke complex. Finally, from published ophiolite studies one is

led to believe that basaltic rocks from ophiolites generally display an increasing degree of pervasive alteration ranging from zeolite facies at the top of the pillow lava to greenschist facies at the bottom of this sequence, to amphibolite facies in the dyke complex. However, the rare detailed petrological studies of metamorphic rocks from ophiolite complexes¹¹ indicate polyphase and more localized metamorphism.

Leg 83 of the Deep Sea Drilling Project (DSDP) has established the most complete reference section to date through the upper oceanic crust, by deepening Hole 504B in the eastern equatorial Pacific to a total depth of 1,350 m below the sea floor (BSF), 1,075.5 m into basement (Fig. 1). Hole 504B had been drilled to 836-m BSF 2 yr previously during Legs 69 and 70, for the purposes of investigating young, geothermally-active ocean crust^{12,13}. In November–December of 1983, Leg 83 re-entered the hole, cored 514 m deeper into basement, and completed an extensive suite of geophysical experiments.

Hole 504B is located in 3,460 m water, 201 km south of the Costa Rica Rift (CRR), the easternmost arm of the Galapagos Spreading Center (Fig. 1). Site 504 is located on magnetic anomaly 3', with an estimated age of 6.2 Myr. Young crust on the southern flank of the CRR passes through the equatorial high productivity zone, with a sedimentation rate of 50 m Myr⁻¹. The 274.5 m of sediment at Hole 504B are dominantly siliceous nannofossil oozes, of late Pliocene age near basement contact. The youngest chert yet found in the oceans occurs at the base of the sediments¹². This chert is thought to have been formed by the high temperature within the thick pile of sediments overlying young oceanic crust¹⁴.

Geothermal setting and temperature measurements

In the vicinity of Site 504, the thick sediment has formed an effectively impermeable seal to convective heat loss from the cooling plate. This is demonstrated both by the pattern of surface heat flow measurements^{15,16}, and by downhole temperatures^{12,17,18}, all of which indicate surface heat flow in agreement with conductive plate cooling predictions of 190–

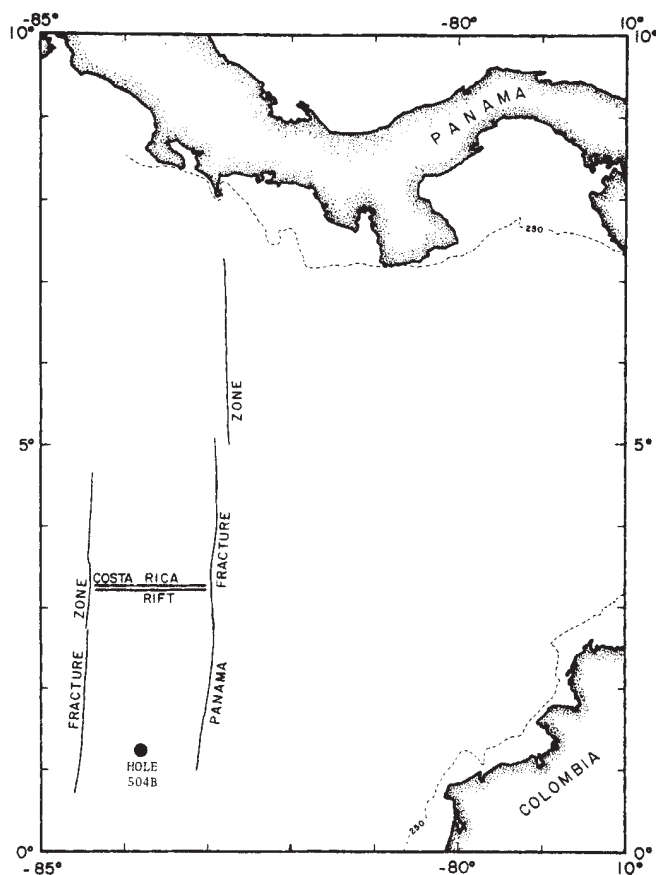


Fig. 1 Location of Hole 504B.

200 mW m^{-2} . Closer to the spreading axis, where the sediment cover is thinner and the basement topography is considerably rougher than at Site 504, surface heat flow values are much less than predicted, presumably due to hydrothermal convection¹⁶.

Downhole temperatures measured on Legs 69, 70 and 83 indicate that an equilibrium, predominantly conductive geothermal gradient holds to the full Hole 504B depth (Fig. 2). However, after Leg 69 drilling punctured the sediment seal, borehole temperatures to a depth of ~360-m BSF were strongly depressed below values measured in the sediments. This was interpreted to indicate flow down the hole of ocean bottom water into the uppermost ~100 m of basement, at a December 1979 rate of $6,000 \text{ l h}^{-1}$ (ref. 17), decreasing to ~1,500 l h^{-1} in November 1981¹⁸. This drawdown is convection forced by basement pore fluid underpressures, which have been attributed to hydrothermal circulation in the basement¹⁹. The basement underpressure originally measured on Leg 69¹⁹ must have significantly rebounded towards the equilibrium hydrostatic pressure after 2 yr of mass flux down the hole into the formation: a mass of seawater of the order of $50 \times 10^6 \text{ kg}$.

Lithostratigraphy

The combined drilling efforts of Legs 69, 70 and 83 have resulted in the penetration in Hole 504B of 1,075.5 m of oceanic basement, with a total recovery of 224 m, corresponding to an average of 20.8%. The lithostratigraphy is shown schematically in Fig. 3 and is summarized below. The upper 575 m of basement consists of intercalated pillow lavas, pillow breccias, hyaloclastites, minor flows, and localized flow and tectonic breccias.

The upper zone is underlain by a 209 m transition zone consisting of pillows, minor flows, and dykes. The upper boundary of the transition zone has been placed at a depth of 846-m BSF, where the first three dykes were encountered within 5 m of each other. Pillow sequences are more abundant, continuous

and heavily brecciated at the top of the transition zone than at the bottom. Fracturing and brecciation are particularly extensive from 911 to 929-m BSF within a large pillow sequence where a mineralized stockwork occurs. Towards the base of the transition zone the pillow units become thinner, and massive units (with a uniform, medium coarse-grained texture, lacking chilled margins) and dykes progressively increase in frequency. The massive units cannot be identified explicitly as dykes because they lack chilled margins.

Dykes were identified by the intrusive nature of the contacts determined in hand specimens and thin sections. Macroscopically, the contacts range from brecciated to razor sharp. In contrast to chilled pillow rims, glass is never encountered in dyke margins and extensive quench morphologies are rare. Dyke margins are best described as having a dense cryptocrystalline to very fine-grained equigranular texture (crystals $< 0.004 \text{ mm}$ in size). Microlites and microphenocrysts are

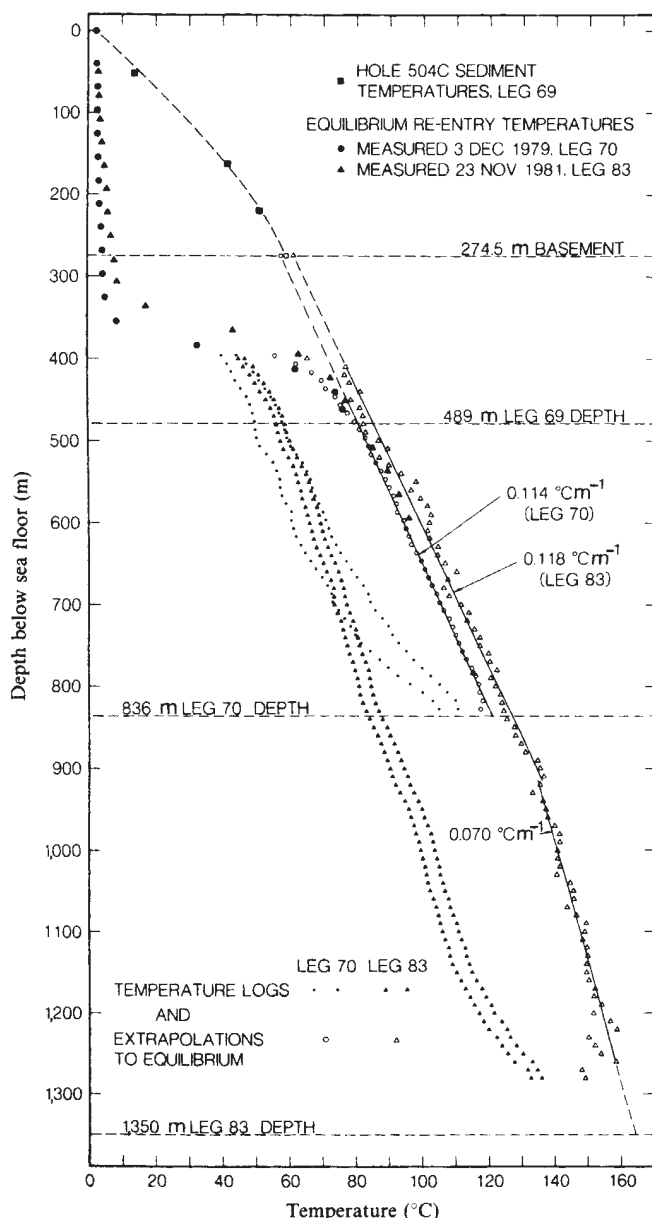


Fig. 2 Borehole temperatures measured on Legs 69, 70, and 83. Equilibrium re-entry temperatures (circles for Leg 70 and crosses for Leg 83) indicate drawdown of bottom water into the upper ~100 m of basement, at a decaying rate. Below this aquifer, extrapolation of temperature logs to equilibrium indicates a conductive heat flow of ~190 mW m^{-2} (from ref. 18.)

nearly always preferentially aligned parallel to the contacts and disaggregated material from the host basalt is also commonly observed at the margins of the intrusive material.

The last recovered pillow was identified at 1,055-m BSF; this depth was chosen by the shipboard petrographers as the boundary of an underlying zone composed predominantly of massive units and dykes in which no pillows were recognized. This unit is interpreted as a sheeted dyke complex, as predicted from ophiolite models of the oceanic crust. The dykes were recovered with one, and rarely two, chilled margins, most of which were steeply inclined (50–60°) to near vertical. Some of these chilled margins are highly brecciated, possibly as the result of forceful dyke intrusions. However, this zone as a whole is far less brecciated than those overlying it. The host material against which the dykes were chilled is always fine to very fine-grained. In contrast, basalts from the inner portions of dykes as a whole tend to have among the coarsest grain sizes recovered (medium coarse-grained, subophitic textures). Although the data are not yet sufficient for a convincing argument, this observation may indicate that dykes tend to intrude predominantly at the margins of previous dykes. This appears to be consistent with observations of one-way chilling documented in ophiolites²⁰. Most of the basalts recovered from the lower zone are regarded as massive. Many of these exhibit significant grain-size variation; that is, fining towards one or both of their implied margins. Such massive units are probably dykes, although (per our definition of a massive unit) no chilled margins were recovered. However, the remainder of the basalts maintained a constant grain-size over the recovered interval and we can only speculate about their mode of emplacement.

The lavas in the uppermost part of Hole 504B are predominantly fine- to medium-grained, olivine + plagioclase ± clinopyroxene ± spinel phryic basalts; aphyric lavas become progressively more common with depth. These basalts can be divided into four petrographic categories on the basis of the phenocryst contents: (1) olivine-plagioclase-clinopyroxene phryic (~29% of the total recovery); (2) olivine-plagioclase phryic (13%); (3) variable olivine-plagioclase-clinopyroxene phryic with accessory chrome spinel (19%); (4) aphyric (40%).

Basement geochemistry

The least-altered basaltic rocks recovered from Hole 504B are remarkably uniform in their major oxide composition. Moreover, no chemical distinction can be made amongst the various petrographic or lithological types. Within the 1,075.5 m basement section, only three lithological units, 14, 18 and 2 m thick, differ significantly from all the other units. These basalts are characterized by higher TiO₂ and P₂O₅ concentrations (1.2–1.4% and 0.14–0.20%, respectively) and by an enrichment of LIL-elements, whereas most of the Legs 69 and 70 basalts are strongly LIL-element depleted²¹.

A general characteristic of the rocks cored during Leg 83 is their high MgO (up to 9.78%) and their very low K₂O content,

which is <0.02%. No systematic downhole variation was observed for the major oxides except MgO and K₂O (see section on alteration). However, there are some minor but irregular variations with depth, which are related to specific lithological units and indicate that limited shallow-depth fractionation processes were operative. The oxide means obtained on Leg 83 rocks are almost identical to those found in Legs 69/70 basalts and their confidence limits in all cases overlap. On a dry weight normalized basis, most rocks analysed vary within the following limits: SiO₂, 49.9% ± 0.9; TiO₂, 0.92 ± 0.1; Al₂O₃, 15.7 ± 0.8; FeO_t, 9.05 ± 0.5; MgO, 8.60 ± 0.6; CaO, 12.9 ± 0.3; K₂O, 0.20 ± 0.1 (Leg 69 section), 0.07 ± 0.04 (Leg 70 section) and 0.02 (Leg 83 section); P₂O₅, 0.08 ± 0.02.

Similar ranges of variation were also obtained on fresh basaltic glasses from the upper part of the hole drilled during Legs 69 and 70. These results suggest that the whole rock analyses (with the possible exception of MgO and K₂O) reflect the primary composition of the rocks and might represent magma compositions. Judging from the high mg values (where $mg = Mg^{2+}/(Mg^{2+} + Fe^{2+})$), which range from 0.62 to 0.70 and average at 0.67, these magmas were rather primitive or unevolved, and had experienced only a small degree of crystal fractionation before their eruption or intrusion. This indicates either a short residence time within the magma chamber and/or the existence of a large magma chamber which was steadily replenished by the same mantle source.

Assuming that the least altered basalts of Leg 83 have similar trace element composition as those from Legs 69/70, it can be concluded that the magmas were derived from a LIL-element depleted mantle source. As a working hypothesis we assume that basalts of the three exceptional units were generated within very small 'pockets' of less depleted mantle material residing below the magma chamber.

Alteration

Alteration of basalts in Hole 504B can be divided into different depth zones based on the presence of various secondary mineral assemblages (Fig. 4). Secondary mineral occurrences from 275 to 585-m BSF are similar to those commonly observed in drilled submarine basalts altered at low temperatures ('submarine weathering'; refs 22–25). Alteration of the rocks in this interval is interpreted to be the result of superimposed stages of oxalic alteration ('iddingsite' and celadonite-nontonite mixtures replacing olivine and filling voids in alteration haloes around cracks) and sub- to anoxic alteration (saponite replacing olivine and filling voids throughout the rocks).

The interval from 528 to 572-m BSF is characterized by the presence of Na-Ca zeolites in relatively frequent thick veins and replacing plagioclase in 1-cm wide alteration haloes around these veins. These zeolite veins and associated wallrock alteration in this zone are interpreted to be the result of a later localized stage of hydrothermal alteration superimposed on the previous alteration at lower temperature²⁶.

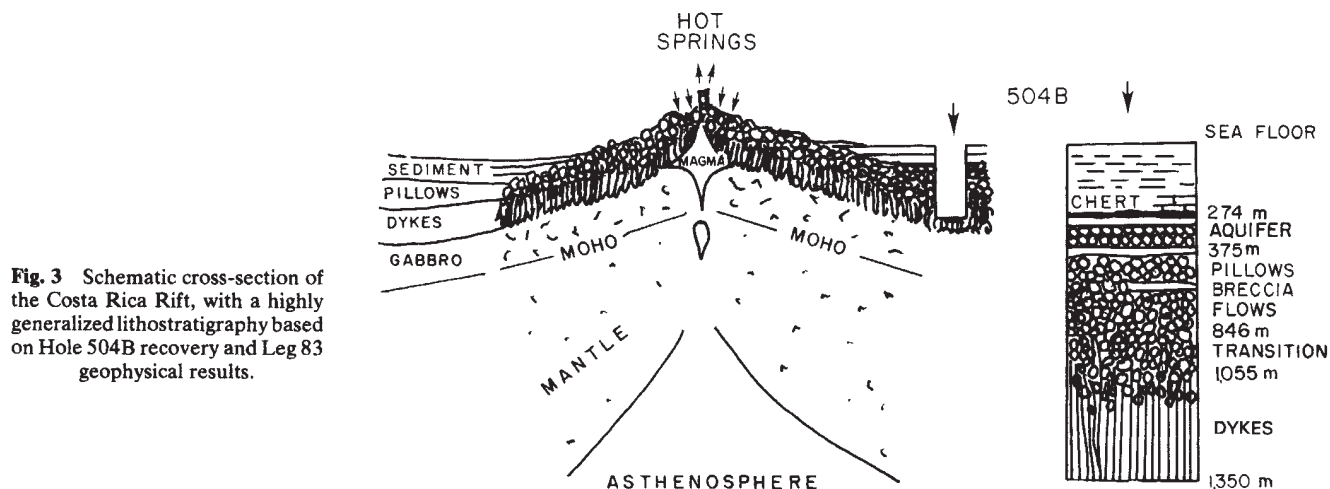


Fig. 3 Schematic cross-section of the Costa Rica Rift, with a highly generalized lithostratigraphy based on Hole 504B recovery and Leg 83 geophysical results.

From 585 to 889-m BSF, alteration is characterized mainly by the occurrence of saponite replacing olivine and filling voids. Secondary minerals in this interval shown in Fig. 4 also occur in veins, with quartz and pyrite abundances generally increasing with depth. Based on mineralogical data and bulk rock oxygen and strontium isotopic data, alteration reactions are interpreted to have occurred in sub- to anoxic conditions either with seawater at low water to rock ratios, or with seawater-derived fluids that had previously reacted with basalt^{26,27}. This stage resulted in the bulk of the alteration observed, characterized mostly by the formation of smectite. Preliminary oxygen isotopic work on isolated smectite and anhydrite suggests that alteration occurred at 60–110 °C (refs 26, 28).

From 889 to 1,350-m BSF, olivine is generally totally replaced by, and interstitial areas filled with chlorite, although smectite or smectite–chlorite mixtures occasionally occur. Titanomagnetite is partly replaced by sphene, and plagioclase is partly replaced by albite, chlorite and minor laumontite. Actinolite occurs as alteration rims of clinopyroxene below 968-m BSF. The above minerals also occur in veins and cementing breccias, whereas the other secondary minerals in this interval (Fig. 4) only occur in veins or breccias.

Based on vein relationships, experimental data²⁹, and analogy with Icelandic geothermal fields^{30,31}, alteration from 889 to 1,350-m BSF is interpreted to have occurred in at least two stages at successively decreasing temperatures. The first stage resulted in the formation of actinolite, chlorite, sphene, albite, pyrite, and possibly epidote and quartz. Temperatures were at least 230 °C, and were >280 °C where actinolite formed (below 968-m BSF). During the second stage, laumontite, scolecite, pyrite, minor amounts of calcite, and possibly chlorite and prehnite formed at temperatures <235 °C. The occasional occurrences of smectite below 889-m BSF remain a problem, however, and may indicate either a lower temperature of alteration superimposed on a greenschist facies stage, or that smectite formed metastably at high temperatures.

A stockwork consisting of abundant veins of chlorite, laumontite, quartz, minor talc, sphalerite, chalcopryrite, and common large crystals (up to 1 cm) of pyrite in highly fractured pillow basalts occurs from 911 to 929-m BSF. Alteration in this stockwork occurred in two stages, similar to those listed above. The abundant sulphides in this zone can be explained either by mixing of a metal, S, and Si-rich hydrothermal fluid with seawater, as is suggested to occur at the Galapagos Spreading Center³², or by a drop in temperature and/or pressure of this solution due to its rise and loss of heat to wall-rock.

Between 1,125 and 1,280-m BSF the rocks are generally less extensively altered and contain fewer and thinner veins than the overlying rocks. Olivine is completely altered to talc, magnetite, and smectite, whereas other igneous minerals are only very slightly altered. The smaller extent of alteration of these rocks may be due to the massive nature of the rocks and the scarcity of veins and cracks which would result in low permeability and restricted circulation of fluids.

From 1,182 to 1,350-m BSF anhydrite occurs in veins, and is interpreted to be a late alteration product. Anhydrite may have formed from heated Ca-enriched seawater in the current thermal regime, as predicted by experimental seawater–basalt reactions³³, or by mixing of hydrothermal fluids with seawater, as at 21 °N on the East Pacific Rise³⁴.

Below 1,280-m BSF basalts are more extensively altered than those in the overlying interval (1,205–1,280-m BSF), and the lowermost rocks recovered are among the most extensively altered.

There is a systematic but slight MgO increase and an associated K₂O decrease with depth as a result of hydrothermal alteration. H₂O+ is generally <1.0% in the least altered rocks from Legs 69 and 70 part of the hole, but shows a maximum (up to 3.4%) between 980 and 1,075-m BSF, coinciding with a zone of extensive mineral replacement; it then drops again towards the bottom of the hole. CO₂ decreases from top (0.1–0.4%) to bottom of the hole, where it is <0.02% in most samples. Note that only the least altered rock samples were analysed, so the chemical changes due to alteration are minimal.

Physical changes down the hole

Leg 83 also conducted the most extensive set of geophysical logs and experiments yet in the Deep Sea Drilling Project (Fig. 5), including packer flow tests, large-scale resistivity³⁵, borehole televiewer and multichannel sonic logging.

Geophysically, the upper oceanic crust sampled at Hole 504B consists of three distinct units. The upper ~100 m of basement is an aquifer of rubbly pillow basalts, breccia zones and a few massive flows. The entire section is fractured but not as extensively as deeper in the hole. Density, thermal conductivity, velocity (both P- and S-waves) and electrical resistivity are all low. Attenuation of seismic body waves along the wellbore and neutron porosity are high, and measured bulk permeability values are as high as those of a good producing oil sand (~60 millidarcies, md). An 'alteration analysis' carried out by cross-correlation of hydrogen-ion-sensitive neutron porosity versus porosity-sensitive γ -ray density logs corroborates Leg 69 core

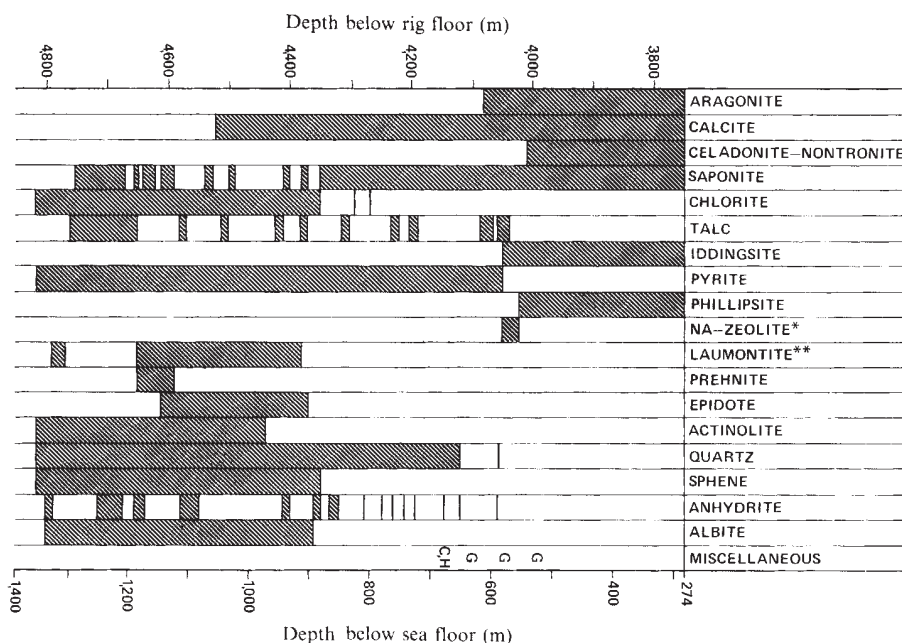
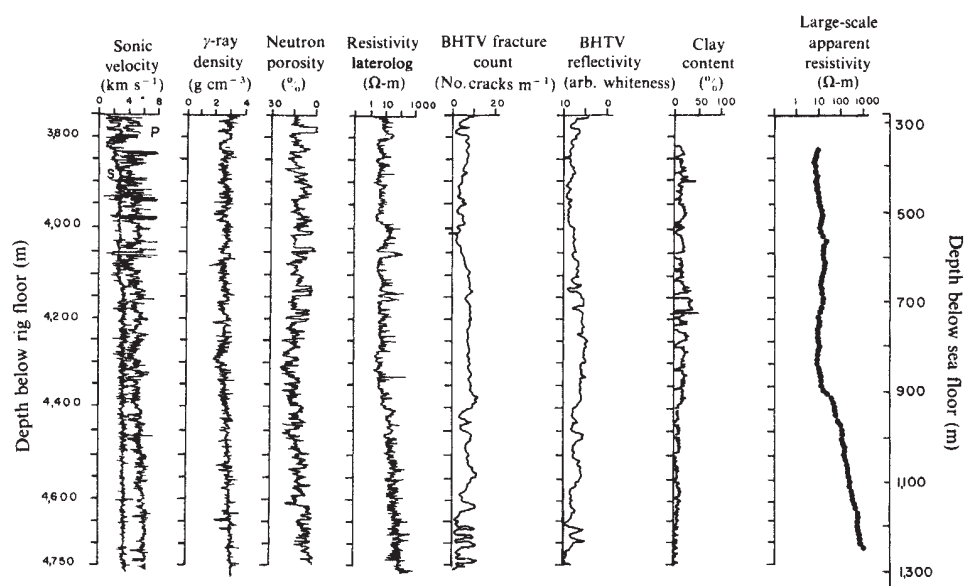


Fig. 4 Occurrences of alteration minerals in basalts recovered from Hole 504B. *, Natrolite, mesolite, thomsonite, analcite, apophyllite; **, including rate scolecite; G, gyrolite; C, chabazite; H, heulandite.

Fig. 5 Geophysical logs obtained from Hole 504B on Leg 83.



analyses¹³ that show only moderate crack-filling by OH^- bearing alteration products such as clays and zeolites. Fractures thus appear to be largely open and filled with seawater³⁶. Under-pressures then turned this upper crust into an active aquifer system when the drill bit penetrated the chert and sedimentary hydraulic lid over this formation. We interpret this zone to be seismic Layer 2A.

From ~100 to 650 m into the basement (375–925-m BSF), increasing gradients in seismic P- and S-wave velocities, electrical resistivity and density, and a decreasing gradient in porosity are observed. Permeability at 475-m BSF has dropped to 4 md. The log cross-correlation alteration analysis shows a steadily increasing bulk content of hydrated minerals, peaking in a zone at 911–929-m BSF, the highly altered, sulphide-rich stockwork.

Borehole televiwer records show a steadily increasing content of fractures (almost all subhorizontal) and a decreasing wellbore reflectivity as the Layer 2A zone of large pillows appears to be replaced by much more extensive brecciation, smaller diameter pillows and fewer massive flows. Shipboard analysis of the cores recovered from this zone could not confirm the decrease in diameter of the pillows as observed in the BHTV records. We interpret this zone to be seismic Layer 2B. Relatively higher seismic velocity seems to be directly related to alteration products filling fractures and voids in this pillow unit³⁶. Thermal conductivity, and magnetic susceptibility and intensity remain constant through this layer. Towards the bottom of Layer 2B, the first dykes are encountered.

Then, at about the depth of the stockwork, all of the physical properties change (Fig. 5). From 875 to 925-m BSF, electrical resistivity, density, and seismic velocity all show a slope change to a higher gradient. Porosity drops; sample thermal conductivities show a stepwise increase of ~25%. Magnetic intensity and susceptibility drop as most titanomagnetite is altered to sphene, but this drop is observed above the level where all other physical properties change. The borehole televiwer shows a drop in the intensity of fracturing and a rise in wellbore reflectivity. The log 'alteration analysis' shows a drop in the bulk content of hydrated minerals at this depth also. Permeability is 0.1 md over the lower 750 m of the hole, a drop of nearly three orders of magnitude. We interpret this to be the transition from seismic Layer 2B to 2C. Below, an increasing number of dykes leads into a sheeted dyke complex towards the bottom of the hole.

At 1,075-m BSF, magnetic intensity and susceptibility increase again as alteration pervasiveness subsides and more titanomagnetite remains intact.

The measured permeability decreases below Layer 2A much more abruptly than was previously expected, because alteration

has plugged the plumbing in Layer 2B. The primary porosity of the rocks forming Layer 2C was already very low before alteration locally reduced it further. We cannot determine whether this permeability stratification has existed since the ridge axis or whether Layer 2A is 'disappearing' upwards as alteration products progressively replace seawater in fractures and pore spaces of the aging upper crust. If the former is the case, it suggests that much shallower, more horizontally-vigorous hydrothermal convection occurs on mid-ocean ridges than was previously thought.

Conclusions

Leg 83 of the DSDP has established the most complete reference section to date through the upper oceanic crust by deepening Hole 504B to a total depth of 1,350 m below the seafloor, or 1,075.5 m into basement. A maximum temperature of 135 °C was measured at the bottom of the hole, estimated to reach ~165 °C after the disturbance due to drilling has decayed. The 274.5-m thick chert-based sediment cover has sealed the crust to convective heat loss, so that if the crust at Site 504 is assumed to have aged through an earlier, hydrothermally-driven cooling phase, then crustal temperatures must have re-equilibrated upwards to present values as the sediment cover sealed off the basement.

The upper ~100 m of the basement consists of a relatively permeable (~60 md) aquifer made up of pillow lavas and breccias, and a few massive flows. Fractures appear to be open and filled with seawater. This is thought to be seismic Layer 2A. From 375 to 850-m BSF more pillow lavas, breccias, and minor massive flows of seismic Layer 2B were encountered, while the frequent fractures in this interval are filled mainly with smectite. The interval 846–1,055-m BSF corresponds to a transition zone with decreasing frequency of pillow lava recovery with depth and correlative increasing dyke recovery. A mineralized stockwork with Fe, Zn and Cu sulphides in laumontite, calcite, quartz, and chlorite veins was recovered in an extensively fractured and brecciated pillow lava sequence within this zone and may be the result of subsurface mixing of hydrothermal fluids with seawater.

The interval from 1,055-m BSF to the bottom of the hole corresponds to part of a sheeted dyke complex, seismic Layer 2C. Just below the mineralized stockwork, a major increase in density, thermal conductivity, velocity (both P- and S-waves) and electrical resistivity occurs. Conversely, the degree of fracturing, porosity and permeability decrease, the latter by nearly three orders of magnitude compared with the overlying formation. Magnetic susceptibility and intensity generally correlate with the extent of alteration of titanomagnetite.

The Layer 2B–2C transition based on physical properties is much sharper than the lithological transition from extrusive rocks to dyke complex (that is, ~50 m compared with ~209 m, respectively). Moreover the boundaries among the various types of alteration zones with depth do not coincide with any lithostratigraphic or physical property limits.

From the sediment–basement interface down to 585-m BSF (representing Layers 2A and 2B), basement alteration resulted from superimposed stages of sub- to anoxic and oxic processes, the latter at relatively high water–rock ratios. These stages resulted in the formation of celadonite–nontronite, ‘iddingsite’, pervasive saponite, and, finally, phillipsite and Ca-carbonates (aragonite, calcite). These processes and products are, on the whole, similar to ‘sea floor weathering’ observed in other DSDP holes. Veins of saponite and Na–Ca zeolites between 528 and 570-m BSF represent a localized later stage of superimposed hydrothermal alteration. Below 585-m BSF down to ~900-m

BSF where greenschist-facies minerals appear, only sub- to anoxic alteration at low water–rock ratios has occurred.

Alteration of basalts below 889-m BSF is interpreted to be the result of at least two stages of alteration: (1) earlier formation of actinolite, chlorite, sphene, albite, pyrite, epidote and quartz at temperatures of at least 230 °C, and >280 °C where actinolite is present (below 969-m BSF); followed by (2) later formation of laumontite, scolecite, pyrite, minor amounts of calcite, and possibly chlorite and prehnite at temperatures <235 °C. Rocks in the interval 1,205–1,280-m BSF are much less extensively altered than over- and underlying rocks, however, due to locally lower permeabilities.

We thank Captain Dill and the crew and technical staff of the DV *Glomar Challenger* for their expertise, especially G. Norrie, A. C. Wheeler, and the drilling crew, who saved the hole on several occasions, leaving it open for possible future re-entry and deeper drilling.

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In situ electrical resistivity and bulk porosity of the oceanic crust Costa Rica Rift

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In situ electrical resistivity was measured in DSDP Hole 504B to a depth of 1,013 m into oceanic basement. Apparent resistivities are about 10 Ω m in about 600 m of pillow lavas, sharply increasing to nearly 1,000 Ω m in the underlying dykes. Bulk porosities calculated from resistivities show a distinct layering, apparently corresponding to geophysical layers 2A, 2B and 2C.

THE circulation of seawater through fresh basalt has a vital role in the development of the chemical and physical nature of the oceanic crust. The modes and effects of circulation are themselves partially controlled by the variable permeability and porosity of the basaltic layer. As these crustal parameters probably depend on irregular fracturing of unknown scale, they cannot be reliably determined from dredged or cored samples.

On the contrary, independent determinations of bulk porosity are essential in relating physical properties measured on recovered samples to the formation properties of the crust. Bulk permeability and porosity must be measured *in situ*, preferably from deep boreholes, at averaging scales large enough to escape the disturbance of drilling on the surrounding formation, as well as to include the effects of irregular fracture porosity.

We report here the results of such an *in situ* experiment, a large-scale resistivity log in the deepest borehole drilled to date into the oceanic crust, Hole 504B of the Deep Sea Drilling Project (DSDP). Over the temperatures encountered in and around the hole (2–165 °C), the electrical conductivity of the pore fluid, essentially seawater, is 1–4 orders of magnitude greater than the intrinsic conductivity of the host basalt. Therefore, although not a direct measurement of porosity, our results were highly sensitive to the bulk porosity of the crust.

Measurement site

Hole 504B is ~200 km south of the Costa Rica Rift (Fig 1), in 6.2 Myr crust under 3,460 m of water. It was cored by three DSDP Legs, 69, 70 (refs 1, 2) and 83 (ref. 3), to a depth of 1,350 m beneath the sea floor, 1,075.5 m into basement (Fig. 2), nearly twice the basement penetration of any other oceanic borehole. Leg 83 clearly penetrated into the sheeted dykes and massive units of layer 2C (ref. 3). Due to the relatively poor core recovery (~20%), good geophysical logs were essential to the interpretation of the section and to relate studies of recovered samples to the bulk properties of the crust.

The 274.5 m of chert-based sediment have sealed the crust at Hole 504B from open circulation of seawater. Surface heat flow measurements⁴ and downhole temperatures^{5,6} indicate dominantly conductive heat transfer through the drilled section, at a value of $190 \pm 10 \text{ mW m}^{-2}$, consistent with predicted plate heat flow. No direct evidence was found for presently active hydrothermal circulation, although a vigorous, pressure-driven⁷ drawdown of bottom water commenced when Leg 69 penetrated the sediment seal^{5,6}. This drawdown is directed through the cased sediment into the upper ~100 m of basement, and it continues at a decaying rate^{3,6}. It has had no discernible effect on the crust deeper than 400 m beneath the sea floor.

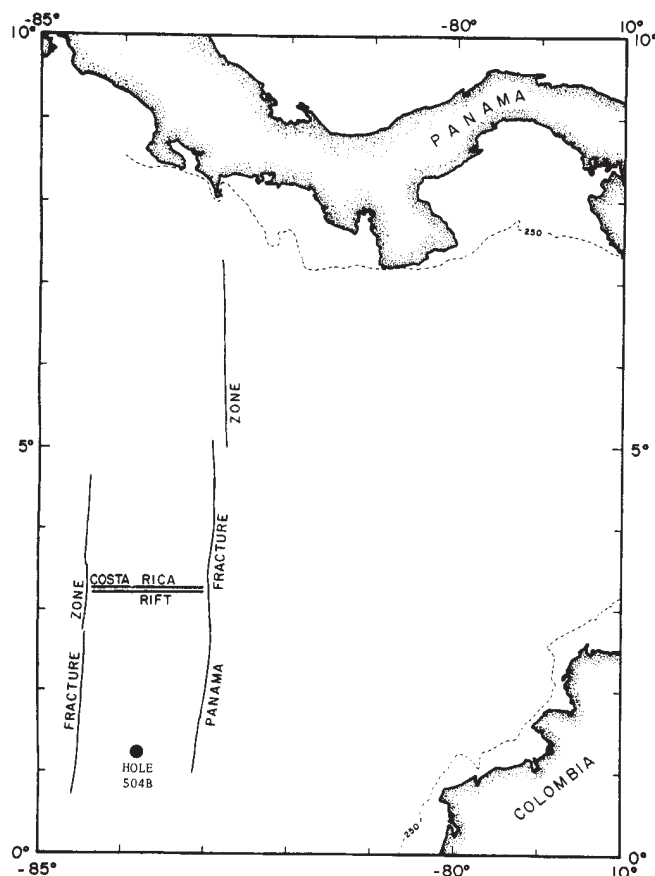


Fig. 1 Location of DSDP Hole 504B in the eastern equatorial Pacific.

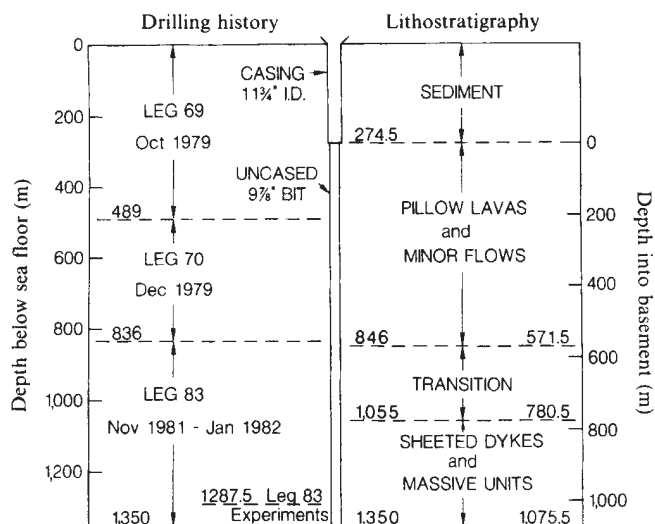


Fig. 2 Hole 504B drilling history (left) and generalized lithostratigraphy (right). The latter is based only on the ~20% core recovery³.

Experimental procedure

The large-scale electrical resistivity experiment was first deployed on Leg 60 by Francis (see ref. 8 for details of the method). It was run twice in Hole 504B, first during Leg 70 to a depth of 836 m (ref. 9), and again during Leg 83 to 1,287.5 m. The experiment is a simple, long-spaced, d.c. resistivity log, for which the downhole configurations are shown in Fig. 3. A large current is passed from the ship down the armoured logging cable, through an insulated cable to a steel sinker-bar/current-electrode. It then passes through the formation and sea back to the ship. Despite the high conductivity of seawater, the long, narrow seawater return path up the borehole is relatively resistive (~5 kΩ km⁻¹), so that the current may be taken to return through the formation⁸. (Nor do the casing or armoured logging cable shunt the current return path⁸.) Due to the resistivity of the formation, a potential gradient is set up in the hole, and it is sampled with several non-polarizing Ag/AgCl electrodes.

The experiment was run at discrete, stationary intervals: at each level, current was passed for about 10 s each under positive and reversed voltage. This allowed removal by averaging of any biases between electrodes due to possible ambient potential gradients in the hole. Currents and potential differences were measured to an estimated 3–5% accuracy on Leg 70, and to 1–3% accuracy with new equipment on Leg 83. On Leg 70, a 500-V power supply passed currents of ~6 A into the formation, and three potential electrodes were spaced 45, 91 and 183 m above the current source (Fig. 3). Potential differences between X and Y electrodes and Y and Z electrodes were recorded and the experiment was run at 25-m intervals. During Leg 83, a 300-V power supply was used, currents ranged over 3.5–4.3 A, and four potential electrodes were spaced 10, 20, 40 and 80 m above the current source. The hole was logged at 10-m intervals, with four potential differences recorded: 1–2, 2–3, 3–4 and 1–4. This redundancy showed excellent Leg 83 data quality: at every level, the sum of the smaller interval voltages duplicated the overall voltage to well within 1%. Although the hole was considerably deeper on Leg 83, a shorter electrode spacing was chosen, partly because the high temperatures in the hole were a threat (unrealized) to melt the insulated cable, and partly to bridge the range of resolution to the much shorter-spaced, focused, commercial resistivity logs also run.

The basic parameters measured were the potential differences between electrodes due to a known power source. Of the nine possible electrode pairs, potential differences were measured for six, and were obtained arithmetically for the other three. Measured voltages were in the 5–50 mV range in the pillow

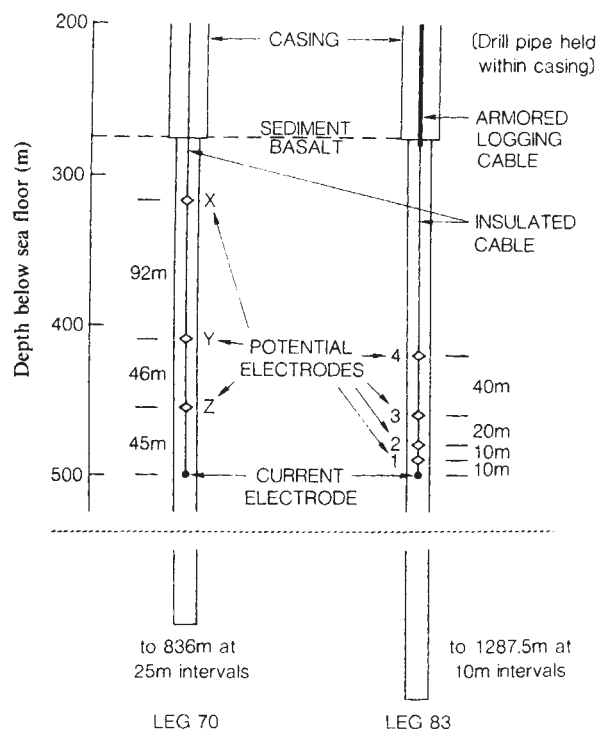


Fig. 3 Electrode configurations for large-scale resistivity experiments in Hole 504B.

lavas logged on Leg 70; when the dykes were logged on Leg 83, voltage differences of 10 mV–10 V were recorded. A 300-m section was repeat-logged on Leg 83; all measured voltages duplicated within 1%.

Apparent resistivity

For a real Earth, with a resistivity varying with depth (and possibly laterally as well), our measured voltage differences are related to the resistivity structure in a complicated, nonlinear fashion. The response of each electrode pair is a function of both the surrounding resistive Earth and of the spacings among potential and current electrodes¹⁰. In effect each electrode pair averages the Earth resistivity over a unique radial and vertical scale. We approximated true resistivities by converting voltage

differences to apparent resistivities (ρ_a), calculated assuming a homogeneous Earth resistivity. With a zero potential assigned to the sea floor, and a current return at the ship, apparent resistivities were obtained by⁸:

$$\rho_a = \frac{2\pi\Delta V}{I} \left\{ \frac{z_2}{h^2 - z_2^2} - \frac{z_1}{h^2 - z_1^2} \right\}^{-1}$$

with h being the depth beneath the seafloor of current source I , and z_1 and z_2 the depths of potential electrodes sensing the voltage difference ΔV ($h > z_2 > z_1$). Since the potential gradient was more sensitive to resistivity variations where the current density was greater, that is closer to the current source, the depths assigned to apparent resistivities were those of the deeper potential electrode.

The electrode spacings were large enough relative to the hole diameter that no geometric borehole correction was necessary to the measurements. Moreover the large-scale experiment investigated far enough into the formation (~5–100 m) to escape the effects of the disturbed zone near the borehole, where temperature, pressure and porosity may have been unrepresentative of *in situ* conditions. We thus interpret our apparent resistivities as radially and vertically averaged *in situ* resistivities, with the poorly-determined averaging scales dependent on the true resistivity structure.

Figure 4 shows apparent resistivities calculated using five of the nine possible electrode pairs. These five pairs shared the same equidimensional ratios of spacings among the current and two potential electrodes (Fig. 3). Thus their averaging characteristics were similar, except that the longer arrays averaged farther into the formation. Indeed the five curves in Fig. 4 are increasingly smoothed, left to right, with increasing array length. The greater detail in apparent resistivities from the shorter-spaced Leg 83 electrode pairs probably reflects better resolution of small-scale vertical variations in resistivity; the smoother resistivities from the longer-spaced electrode pairs are probably more representative of the bulk properties of the crust.

The apparent resistivities in Fig. 4 are all consistent with bulk crustal resistivities on the order of 10 Ω m through the ~600 m of pillow lavas cored in Hole 504B. Below this level apparent resistivities increase sharply, approaching 1,000 Ω m in the sheeted dykes and massive units of layer 2C. As occurred with all other geophysical measurements in the hole, the transition in apparent resistivities is much sharper than the lithostratigraphical transition zone of ~209 m (Fig. 2) determined from

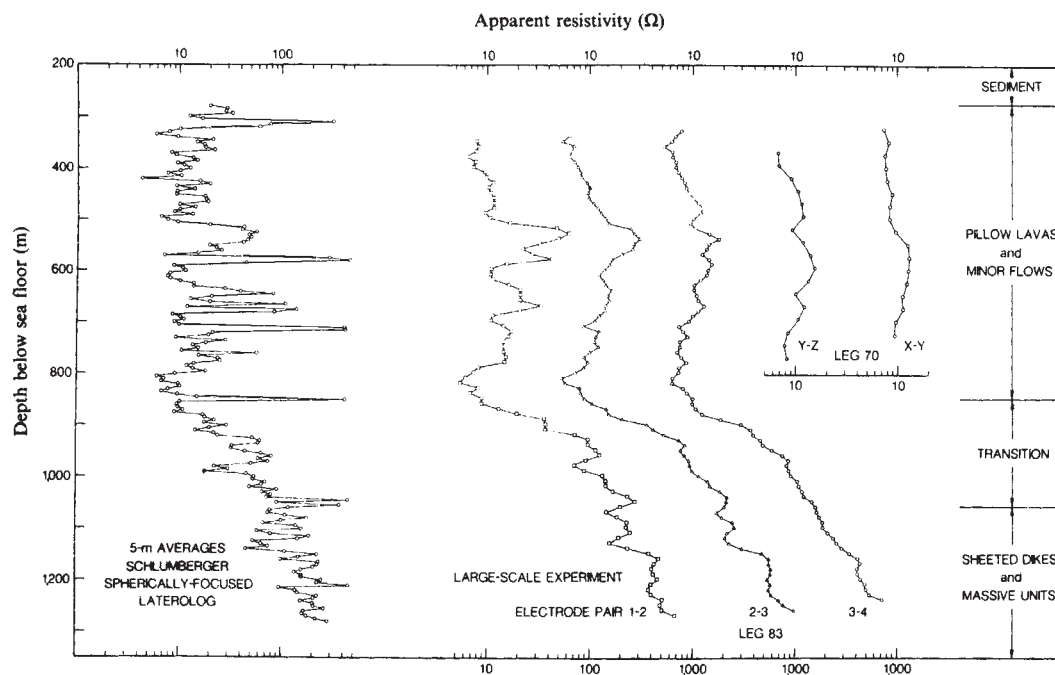


Fig. 4 (Left to right) 5-m averages of borehole-corrected resistivities measured by Schlumberger logging tool, five sets of apparent resistivities from the large scale experiment, and generalized lithostratigraphy of Hole 504B. Note logarithmic scales for resistivities, which are near 10 Ω m in the upper basement and approach 1,000 Ω m in the deepest dykes.

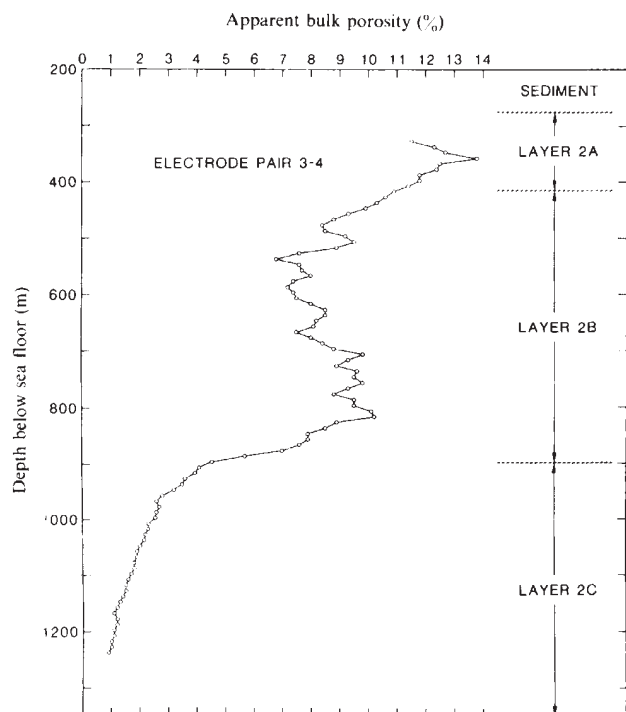


Fig. 5 Apparent bulk porosities calculated from apparent resistivities determined from electrode pair 3–4, using Archie's law with a temperature-corrected seawater resistivity: $\rho_a = \rho_{sw}\phi^{-2}$. On the right, divisions between layers 2A, 2B and 2C have been picked arbitrarily, based on changes in the apparent porosities shown here.

recovered cores⁴. Finer scale variations in apparent resistivities (and our later porosity estimates), particularly within the pillow lavas, may be related to the variable degree of alteration³, either by partial filling of pore spaces, or because of the enhanced conductivities of alteration products. Comparison of our *in situ* resistivities with typical values of 5–3,000 Ω m measured on basalt samples^{11–13} suggests a significant fracture porosity in the pillow lavas, sharply decreasing into the dykes below.

Considerably greater detail in apparent resistivities is shown by the much smaller-spaced Schlumberger spherically-focused laterolog (SFL) run on Leg 83, even when these borehole-corrected readings were averaged over 5 m (Fig. 4). As the radius of investigation of the SFL is relatively small (~ 0.5 m), some of this apparent detail is not directly representative of *in situ* conditions, but instead reflects some combination of exaggerated tool response at resistivity contrasts and the effect of the disturbed zone near the borehole. Nevertheless, average SFL resistivity values agree reasonably well with the large-scale values, except in the deeper, highly resistive section. We attribute this discrepancy to the greater contrast in the deeper section between the resistivity of the disturbed zone and *in situ* resistivity, as well as to possible errors in borehole-correcting the SFL response for the seawater in the hole. We take the large-scale resistivity values to be much better representative of *in situ* resistivity.

Bulk porosity from apparent resistivity

In commercial well logging, the porosity, ϕ , is often obtained from the measured resistivity, ρ_m , and the resistivity of the pore fluids, ρ_f , by application of the empirical Archie's law¹⁴:

$$\frac{\rho_m}{\rho_f} = a\phi^{-m}$$

with $a \sim 1$, $m \sim 2$. Although this relationship was developed for sedimentary rocks of relatively uniform porosity¹⁵, it has been applied with reasonable success to samples of both oceanic^{11–13} and continental^{16,17} basement. We cannot rigorously justify application of Archie's law to *in situ* resistivity

measurements in the oceanic crust, particularly because of the three-fold nature of the porosity¹⁸: electrical conduction may be due to vesicular (pore), crack or fracture porosity. The form of the resistivity–porosity relationship depends on the nature of the porosity: exponents near but possibly >2 have applied to measurements on basalt samples with dominantly grain-boundary (microcrack) porosity^{11–13,16,17}, whereas exponents <2 have applied in interpreting logging measurements in the fractured upper levels of oceanic basement^{19,20}.

As Hole 504B clearly penetrated through the highly fractured and partially sealed pillow lavas, into relatively unfractured and less altered dykes³, we cannot realistically expect a single resistivity–porosity relationship to hold throughout. Nevertheless, we roughly approximated bulk porosities by applying Archie's law, compromising on an exponent of 2. The salinity of the pore fluids closely corresponded to that of seawater²¹, so we used the temperature-sensitive resistivity of seawater. Based on laboratory measurements^{22–24}, we applied a linear relationship⁹ of seawater conductivity, σ_{sw} ($S\ m^{-1}$), with temperature, T ($^{\circ}C$),

$$\rho_{sw}^{-1} = \sigma_{sw} = 3 + T/10$$

over the $\sim 160^{\circ}$ range of equilibrium crustal temperatures⁶.

Figure 5 shows apparent bulk porosities calculated using data from the longest-spaced Leg 83 electrode pair, 3–4, which yielded the largest-scale averages of *in situ* resistivity. Immediately obvious is a striking stratification of porosity, directly corresponding to layers 2A, 2B and 2C, as interpreted from other geophysical measurements in the hole³. We obtained bulk porosities of ~ 12 – 14% in the highly permeable aquifer, about 100 m thick, identified as a thin layer 2A (ref. 3), 7– 10% in the deeper pillows that form layer 2B, and $<3\%$ in the dykes and massive units of layer 2C. These values are lower than porosity estimates of 13% or higher obtained from logging layer 2B in Atlantic DSDP holes in older crust^{19,20}.

A borehole seismic experiment²⁵ in Hole 504B showed a clear layer 2B/2C break, consistent with our sharp porosity change, but it showed no evidence for layer 2A. However, a ~ 100 -m thick layer 2A may be beyond the resolution of both that experiment and conventional refraction techniques²⁶. The Leg 83 sonic logs (Fig. 5 of ref. 3) are not inconsistent with our three-layer stratification of apparent bulk porosity.

Shipboard measurements of grain-boundary porosities of homogeneous basalt samples from Hole 504B yielded mean values of $5 \pm 2\%$ in the pillows of layer 2A+2B (refs 12, 13), $3 \pm 2\%$ for the entire Leg 83 section, and $\sim 1\%$ in the deeper dykes²⁷. Thus our results corroborate interpretations based on other Leg 83 measurements^{3,28}, namely that: (1) a significant, unsealed fracture porosity exists in layer 2A; (2) fracture porosity is partially sealed by alteration products in layer 2B; and (3) very little fracture porosity exists in the dykes of layer 2C. This low layer 2C fracture porosity may have resulted from sealing by compression or infilling by alteration products. An alternative possibility must be considered—that the dykes may always have been relatively unfractured, which would probably require that hydrothermal circulation never penetrated significantly into layer 2C. In support of this hypothesis, we note that both fracture intensity and degree of alteration, determined *in situ* and from recovered samples, drop significantly in the dykes^{3,28}.

While we have no firm basis for confidence in our values of bulk porosity, we are confident that the porosity stratification revealed by applying Archie's law is not an artefact of the resistivity–porosity model used. In particular, the hundred-fold increase of resistivity into the dykes strongly implies a sharp reduction in bulk porosity, to 1% or lower. This result is consistent with the interpretation of a sea floor electromagnetic experiment on the flank of the East Pacific Rise at $21^{\circ}N$, which suggests a similar increase of resistivity at a depth of ~ 1.4 km (ref. 29). In both that experiment and ours, the remarkable result was the surprisingly shallow level at which apparent resistivity sharply increases, and, by inference, fracture porosity

and permeability to hydrothermal circulation decrease significantly.

Conclusions

We have successfully measured *in situ* electrical resistivity throughout nearly 1 km of layer 2 of the oceanic crust at Hole 504B. Crustal resistivities are about 10 Ω m in the pillow lavas forming layers 2A and 2B, and strongly increase to nearly 1,000 Ω m in the dykes and massive units of layer 2C. Bulk porosities estimated from crustal resistivities indicate three porosity layers directly corresponding to layers 2A, 2B and 2C. Our results suggest that there is almost no fracture porosity in layer 2C. The sharp increase with depth in apparent resistivity, and order of magnitude decrease in apparent bulk porosity, are

highly consistent with the steep reduction with depth in bulk permeability also observed in the hole^{3,7,30}. An important implication of the significant reduction in fracture porosity and permeability with depth, particularly into the dykes, is that any hydrothermal circulation must presently be limited to shallow crustal levels on the flank of the Costa Rica Rift (~1 km), or mostly confined to the pillow basalts. We speculate that hydrothermal circulation may have been limited in this way closer to the spreading axis.

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Construction of influenza haemagglutinin genes that code for intracellular and secreted forms of the protein

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The DNA sequences encoding the amino-terminal signal peptide or the carboxy-terminal hydrophobic anchor have been deleted from a cloned gene coding for the haemagglutinin (HA) of influenza virus. The wild-type gene has previously been shown to be expressed with high efficiency from simian virus 40 (SV40)-HA recombinant vectors into a fully glycosylated protein that is displayed on the infected cell's surface in an antigenically and biologically active form. The anchor-minus HA also is glycosylated but is secreted efficiently into the medium. By contrast, the signal-minus HA is produced only at low levels, is not glycosylated and is located intracellularly.

HYDROPHOBIC amino acid sequences have a crucial role in determining the biosynthetic pathway and ultimate destinations of secretory and integral membrane proteins of eukaryotic cells. The precursors of such proteins invariably contain at or very near their N-termini a tract of hydrophobic amino acids which functions in the translocation of the nascent polypeptide across the lipid bilayer of the endoplasmic reticulum¹⁻⁶. In addition, the majority of membrane proteins have at their C-termini another hydrophobic region that serves to anchor the completed protein into the lipid bilayer⁶⁻¹⁰. Analyses of both types of sequences from a large number of proteins have revealed a remarkable diversity of amino acid sequence, although hydrophobicity is always maintained. These results suggest that the essential difference between a secretory and an integral membrane protein is the presence or absence of an anchoring C-terminal hydrophobic peptide; and they raise the possibility that removal of the signal sequence might divert a secreted

protein from its normal biosynthetic pathway into the cytoplasm of the cell.

Influenza virus haemagglutinin (HA) is the best characterized of all integral membrane proteins. Its complete amino acid sequence has been deduced^{6,11-16} and its three-dimensional structure¹⁷, organization into trimeric structures^{17,18} and orientation with respect to the membrane¹⁹ have been determined. In addition, the location of its major antigenic sites²⁰ and the points at which the protein is glycosylated^{6,17} have been defined. Apart from the specialized features associated with its antigenicity and biological roles of attachment²¹ and penetration^{22,23}, the structure of HA is characteristic of the major class of cellular integral membrane glycoproteins, containing typical terminal hydrophobic regions.

Recently the complete RNA gene for HA from the A/Japan/305/57 strain of influenza virus was converted to double-stranded DNA, cloned, sequenced and inserted into a

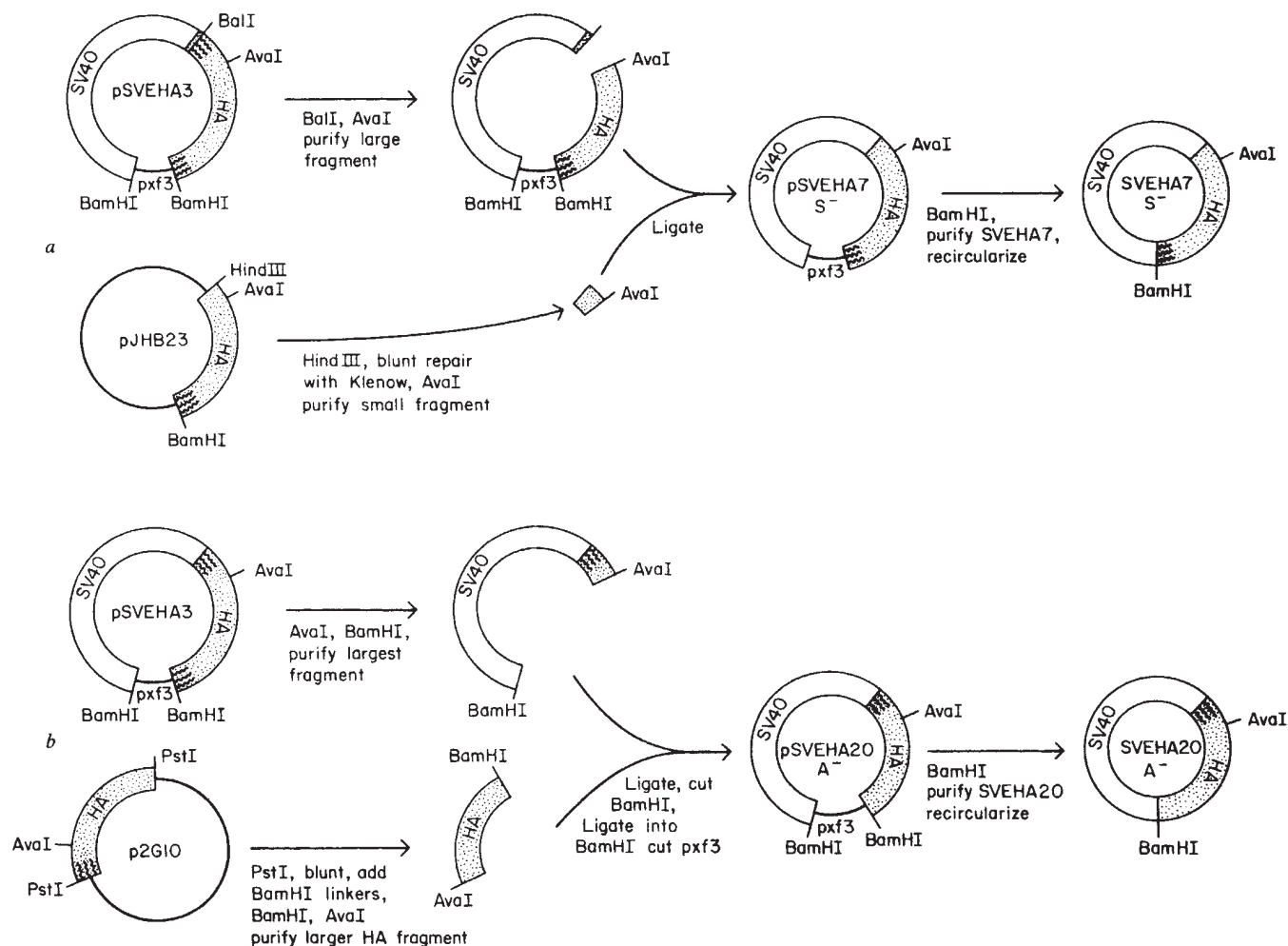


Fig. 1 Construction of recombinant vectors containing mutant genes that lack nucleotide sequences coding for the terminal hydrophobic regions of HA. *a*, The construction of SVEHA7-S⁻ (signal-minus) used two previously synthesized plasmids: pSVEHA3 which consists of the late replacement vector SVEHA3²⁵ (wild-type HA sequence) cloned into plasmid pXf3 (ref. 28), and pJHB23 which contains a truncated HA gene that was created by *Bal*31 digestion of the wild-type gene²⁵ and lacks the nucleotides coding for the signal peptide. pSVEHA3 was digested with *Bal*I and *Ava*I and the larger fragment was purified by polyacrylamide gel electrophoresis. (Restriction endonuclease digestions were carried out using the conditions recommended by New England Biolabs. Other cloning procedures were done as described in Maniatis *et al.*²⁸.) pJHB23 was digested with *Hind*III and the protruding tails were filled in using the Klenow fragment of DNA polymerase I. The plasmid was then further digested with *Ava*I and the smallest restriction fragment was purified by polyacrylamide gel electrophoresis. The two fragments were ligated using T4 DNA ligase and transfected into *E. coli* DH-1. A plasmid, pSVEHA7-S⁻, that had the desired structure was identified by restriction endonuclease digestion and the extent of the deletion was verified by determination of the nucleotide sequence across the junction⁴⁹. This plasmid was then digested with *Bam*HI and the SV40-HA fragment was purified by gel electrophoresis and circularized by ligation at low DNA concentration (3 μ g ml⁻¹) to produce the recombinant vector SVEHA7-S⁻. *b*, The construction of SVEHA20-A⁻ (anchor-minus) also used pSVEHA3, together with the plasmid p2G10 that lacks the HA nucleotide sequences coding for the C-terminal anchor peptide⁶. pSVEHA3 was digested with *Ava*I and *Bam*HI and the larger fragment was purified. p2G10 was digested with *Pst*I, the protruding tails were removed using S₁ nuclease and *Bam*HI linkers (Collaborative Research) were added. After digestion with *Bam*HI and *Ava*I, the 1,300-nucleotide HA fragment was purified. The two fragments were ligated and transfected into *E. coli* DH-1. A plasmid, SVEHA20-A⁻, that had the desired structure was identified and verified as described above, and the recombinant vector, SVEHA20-A⁻, was produced by purification and ligation.

variety of prokaryotic and eukaryotic expression vectors^{6,24-27}. When this version of the HA gene is introduced into eukaryotic cells using vectors derived from SV40, it is expressed as a protein whose structure and biological properties are indistinguishable from those of authentic HA²⁵. These cloned, expressing copies of the HA gene provide the material to analyse the effects of mutations on the structure and function of the molecule. Our initial experiments have focused on an analysis of deletion mutants of HA that lack the sequences coding for the N- or C-terminal hydrophobic peptides.

Construction of deletion mutants

The late replacement vector SVEHA3 consists of the coding sequences of the wild-type HA gene inserted into the late region of SV40²⁵. This vector was used as the basis for the construction of two new vectors containing mutant HA genes that lack

sequences coding for either the N-terminal or C-terminal hydrophobic peptides of the protein. In neither case did the deletions affect the placement of the HA coding sequences with respect to the upstream, controlling SV40 sequences. The construction of these mutants is described in Fig. 1. The signal-minus mutant (SVEHA7-S⁻) was derived from a plasmid (pJHB23) that contains a HA gene from which the 5' non-translated sequences, the initiation codon and the nucleotides coding for the signal peptide had been removed by digestion with exonuclease *Bal*31 (ref. 25). A restriction fragment containing these truncated 5' sequences was used to replace the sequences of the wild-type late replacement vector (pSVEHA3) lying between the *Bal*I site immediately following the ATG of the HA gene and the *Ava*I site at nucleotide 340 (ref. 6). This resulted in a mutant HA gene in which the initiation codon was fused in phase to the codon for the first amino acid of the

Table 1 Comparison of the properties of wild-type and mutant HAs produced from SV40-HA vectors

	SVEHA3 Wild-type	SVEHA20-A ⁻ Anchor-minus	SVEHA7-S ⁻ Signal-minus
Location	Cell surface	Secreted to medium	Intracellular
<i>M_r</i> (subunit)	75,000	72,000	63,000
Structure	Trimer	Trimer	?
Glycosylation	+	+	-
Red cell binding to infected cells	+	-	-
HA activity in cell extracts or medium	+	-	-
Cell-cell fusion (low pH dependent)	+	-	-
Molecules per cell (60 h post-infection)	6 × 10 ⁸	1 × 10 ⁹	1 × 10 ⁶
Amount produced per 10 cm dish (Extract)	200 µg	28 µg	0.5 µg
(60 h post-infection) (Medium)	0	300 µg	0

The subunit structure of the purified proteins was determined by cross-linking with dimethyl suberimide¹⁸. Red cell binding was assayed as described previously²⁵. Cell-cell fusion was measured by the method previously described²³.

mature HA1 polypeptide (Fig. 2). The remainder of the HA coding sequences were unaltered. The anchor-minus mutant (SVEHA20-A⁻) was derived from a plasmid (p2G10) containing a copy of the HA gene that lacks sequences coding for the C-terminal hydrophobic peptide and the putative cytoplasmic tail of the protein⁶. An *Ava*I-*Bam*HI restriction fragment from this truncated gene was used to replace the corresponding wild-type HA sequence in pSVEHA3 (Fig. 1).

The mutant forms of the late replacement vectors were cloned in plasmid pXf3 (ref. 28) and propagated in *Escherichia coli*. The novel nucleotide sequences formed as a consequence of the deletions were determined and the amino acids encoded by the altered regions are shown in Fig. 2. The HA protein encoded by SVEHA7-S⁻ lacks 14 amino acids that comprise the wild-type signal peptide. These sequences are replaced by a tract of three amino acids—Met, Glu, Leu—that is clearly neither long enough nor sufficiently hydrophobic to function as a signal peptide. On the other hand, the protein that is encoded by SVEHA20-A⁻ lacks 38 amino acids from the C-terminus of the wild-type HA, including the hydrophobic anchoring sequence and the short hydrophilic tail. These residues are replaced in the mutant by 11 predominantly charged amino acids encoded by nucleotides from the GC homopolymer tails, the *Bam*HI linker and a short stretch of SV40 sequence which precedes the first in-frame termination codon.

When the sequences of the mutants had been verified, the SVEHA recombinant genomes were excised from their plasmids by digestion with *Bam*HI, purified by gel electrophoresis and recircularized by ligation at low DNA concentration²⁵. The resulting circular DNA molecules were then introduced together with a helper virus genome, dl1055 (ref. 29), into CV-1 cells using DEAE-dextran as the facilitator³⁰. Virus stocks were prepared as described previously²⁵.

		signal cleavage
SIGNAL*	NH ₂ - Met, Ala, Ile, Ile, Tyr, Leu, Ile, Leu, Leu, Phe, Thr, Ala, Val, Arg, GLY, ASP, GLN, ILE, CYS, ILE - HA1	
SIGNAL*	-15 -10 -5 -1 1 5	
SIGNAL*	NH ₂ - Met, Glu, Leu, GLY, ASP, GLN, ILE, CYS, ILE - HA1	
ANCHOR*	HA2 - LYS, LEU, SER, SER, MET, GLY, VAL, TYR, GLN, Ile, Leu, Ala, Ile, Tyr, Ala, Thr, -	***
ANCHOR*	502 - LYS, LEU, SER, SER, MET, GLY, VAL, TYR, Pro, Pro, Gly, Ser, Arg, His, Asp, -	502
ANCHOR*	-Val, Ala, Gly, Ser, Leu, Ser, Leu, Ala, Ile, Met, Met, Ala, Gly, Ile, Ser, Phe, -	
ANCHOR*	-Lys, Ile, His - COOH	520
ANCHOR*	***	
ANCHOR*	-Trp, Met, Cys, Ser, Asn, Gly, Ser, Leu, Gln, Cys, Arg, Ile, Cys, Ile - COOH	547

Fig. 2 Comparison of the amino acid sequences coded by wild-type and mutant HA genes. The novel nucleotide sequences in the mutant HA genes, formed as a consequence of the deletions, were determined by the Maxam-Gilbert technique⁴⁹. The amino acid sequences in the altered regions are compared with the wild-type sequence. Residues that are conserved between mutant and wild-type proteins are shown in capital letters, while those that differ are indicated by lower case letters. *** Indicates the probable beginning and end of the C-terminal hydrophobic sequence that is inserted in the lipid bilayer.

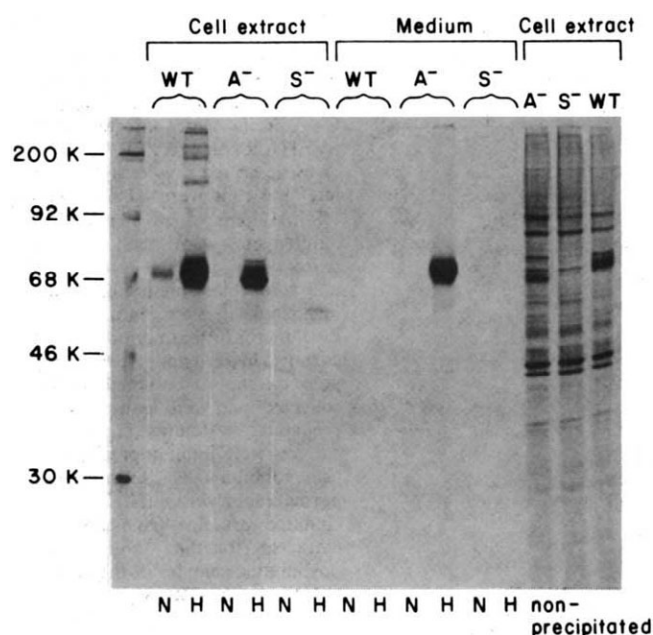
Expression of variant forms of HA from SV40-HA recombinants

Monolayers of CV-1 cells were infected at high multiplicity with vectors carrying the wild-type and mutant forms of the HA gene. Sixty hours later the media above the monolayers were collected and cell extracts were prepared²⁵. The amount of HA antigen in aliquots of the supernatants and cell extracts was measured by a solid phase radioimmunoassay²⁵ and the results are shown in Table 1. HA expressed from the SVEHA3 vector is found exclusively in a cell-associated form: previous work²⁵ has shown that it is anchored into the plasma membrane. By contrast, the anchor-minus HA expressed from SVEHA20-A⁻ is found in the medium in very high amounts, indicating that it is secreted from infected cells. The small fraction of HA antigen found in the cell extract may represent nascent protein that is still being transported within the cell or secreted material that has adsorbed to the cell surface. Both the wild-type and the anchor-minus HA are very efficiently expressed from the recombinant vectors, with up to 10⁹ molecules of HA synthesized in each infected cell. Once the infection is well established (~20 h post-infection) serum-containing medium is no longer necessary for the continued production and secretion of HA. Thus, at 60 h after infection, 40 h after changing to serum-free medium, some 300 µg of essentially pure HA can be collected from a single 10-cm plate of cells infected with the anchor-minus mutant. Much less HA antigen is synthesized in cells infected with SVEHA7-S⁻. Only 0.5 µg of HA is produced per dish, all of which is cell-associated.

Characterization of HA mutants

Supernatants and extracts obtained from cells radiolabelled with ³⁵S-methionine between 48 and 49 h after infection were immunoprecipitated and analysed by SDS-polyacrylamide gel electrophoresis (PAGE). Figure 3 shows the effects of the mutations on the size and location of the HA protein. As described previously²⁵, cells infected with SVEHA3 express a protein of molecular weight (*M_r*) 75,000 that is specifically precipitated by anti-HA serum from cell extracts but not from the supernatant medium. However, the HA protein synthesized during the 1-h labelling period by cells infected with SVEHA20-A⁻ can be precipitated from both the cell extract and the medium, thus confirming that it is secreted from the cells. Both the wild-type and secreted forms of HA can be seen as major bands when the proteins in the labelled extracts are separated by SDS-PAGE without previous immunoprecipitation (Fig. 3). The slightly lower molecular weight of the secreted form of HA is as expected because the mutant gene encodes a protein that is 27 amino acids shorter than the wild-type molecule (Fig. 2).

A smaller protein, produced in much reduced quantities, is precipitated from extracts of cells infected with SVEHA7-S⁻. In size, this protein is the same (*M_r* 61,000) as the non-glycosylated HA synthesized *in vitro* by translation of wild-type HA

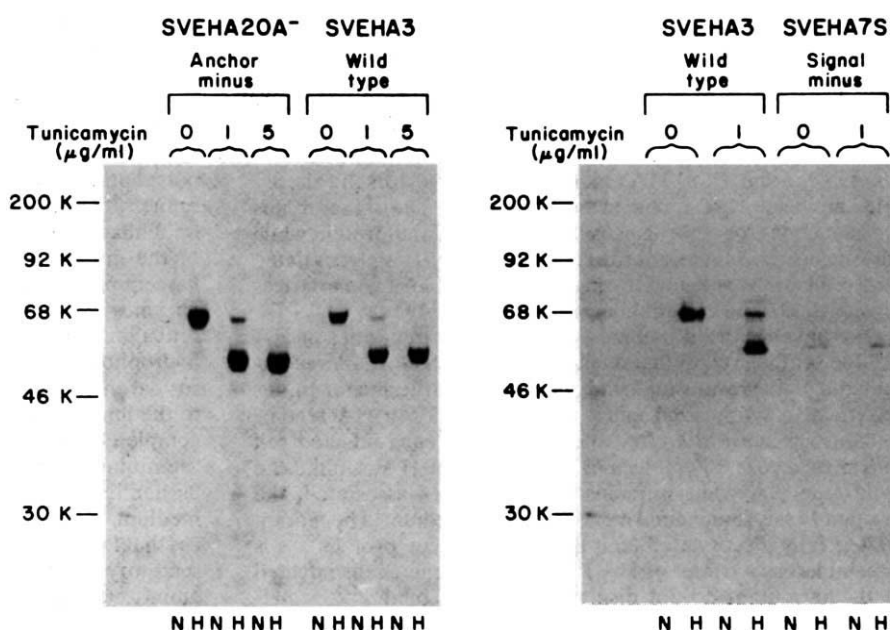


mRNA²⁵. This result is consistent with synthesis of the signal-minus HA as a cytoplasmic protein on free polyribosomes. The failure to detect a major band of M_r 61,000 in the non-precipitated sample indicates that the decreased amount of protein observed both in this experiment and in the solid-phase radioimmunoassay is not due to the inability of the anti-HA serum to recognize the non-glycosylated form of the protein. Restriction endonuclease analysis of viral DNA extracted³¹ from cells infected with the various recombinant viruses indicated that the numbers of recombinant genomes were comparable in cells infected with the anchor-minus, signal-minus and wild-type SV40-HA recombinants. However, Northern hybridization³² of RNA purified from cells infected with SVEHA7-S⁻ showed

that the level of HA-specific sequences was several-fold lower than in cells infected with the other recombinants. Furthermore, experiments in which infected cells were labelled for short times with ^{35}S -methionine and then chased showed that the signal-minus HA was more rapidly degraded than the wild-type protein. Taken together, these results indicate that the low yield of signal-minus HA may be due to instability of both the mutant mRNA and its protein product.

Analysis of glycosylation of HA mutants

To ascertain whether the various HA species were glycosylated, infected cells were labelled with ^{35}S -methionine in the presence of tunicamycin, a drug that blocks the formation of the dolichol-oligosaccharide donor and thus prevents primary glycosylation of the nascent polypeptide³³. SDS-PAGE analysis of the resulting proteins is shown in Fig. 4. Tunicamycin treatment of cells infected with SVEHA3 or SVEHA20-A⁻ resulted in a reduction in the sizes of the immunoprecipitated proteins to M_r s of 61,000 and 58,000, respectively. These sizes are the same as those of the non-glycosylated forms of the protein synthesized *in vitro* by translation of wild-type and anchor-minus HA mRNA (data not shown). A small proportion of the non-glycosylated form of the anchor-minus protein was detected in the medium (data not shown), indicating that glycosylation is not an absolute requirement for secretion. The molecular weight of the HA variant synthesized in cells infected with SVEHA20-S⁻ was unaffected by tunicamycin treatment. This result confirms that signal-minus HA does not become glycosylated.



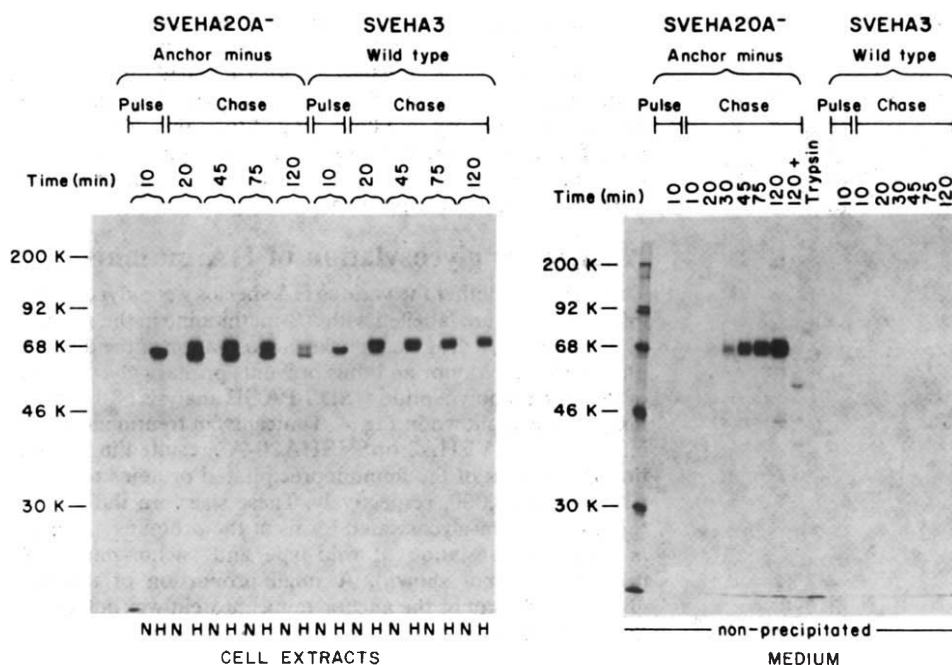


Fig. 5 Rate of processing of wild-type and anchor-minus HA glycoproteins. Monolayers of CV-1 cells were infected with SVEHA20A⁻ or SVEHA3 viruses as described previously²⁵. 48 h later the cells were labelled with ³⁵S-methionine²⁵ for 10 min. The monolayers were then washed twice with medium containing nonradioactive methionine and incubated at 37 °C in complete medium for further periods of time as shown in the figure. At each time point the supernatant medium was collected and cell extracts were prepared²⁵. Aliquots of the cell extracts were immunoprecipitated using rabbit anti-HA serum (H) or normal rabbit serum (N). The precipitated proteins from the cell extracts, together with non-precipitated samples of the supernatant media, were separated by SDS-PAGE and autoradiographed²⁵.

glycosylation occurs. This completion of glycosylation of the wild-type HA takes ~20 min. By contrast, the transition between the sharp and diffuse forms of the anchor-minus HA in the cell extract occurs over a more protracted period. The radiolabelled protein first appears in the medium after 20–30 min and labelled material continues to be secreted for over 2 h. Two lines of evidence indicate that only terminally glycosylated HA reaches the surface of infected cells: first, only the diffuse HA bands are susceptible to digestion by trypsin added to the medium (results not shown) and second, only the diffuse band of the anchor-minus mutant is secreted from the cells (Fig. 5).

The same results were obtained when these pulse-chase experiments were repeated using tritiated mannose, glucosamine and fucose as precursors to follow the biosynthesis and maturation of the HA molecules (results not shown). Furthermore, it was apparent that although the time course of addition of the peripheral sugars was altered, the final composition of the oligosaccharide side chains of the completed HA molecule was unaffected by the deletion of the C-terminus of the protein.

Discussion

As a first step towards a detailed analysis of the role of the hydrophobic regions of HA, we have constructed mutants by deleting from the cloned gene DNA sequences that encode either the N- or C-terminal amino acid sequences of the protein. When these signal-minus or anchor-minus mutants are expressed from SV40-HA recombinant viral vectors the altered HA molecules are found in new locations. The signal-minus variant codes for a non-glycosylated, intracellular protein while the anchor-minus variant specifies a protein that is glycosylated and efficiently secreted from the cell. Table 1 summarizes properties of the mutant and wild-type HAs.

We have reported previously that copious quantities of HA can be synthesized in mammalian cells from SV40-HA recombinants²⁵. The results reported here show that the anchor-minus HA is also synthesized efficiently from an SV40-HA recombinant. By late times after infection (60 h), each infected cell has produced ~10⁹ molecules of the mutant HA. Unlike the wild-type HA which remains exclusively cell-associated, the mutant HA is found almost entirely in the medium. The mutant HA is fully glycosylated and, like the wild-type protein^{17,18}, is assembled as a trimer of HA subunits. However, cells infected with the mutant do not display erythrocyte binding²⁵ or pH-dependent cell fusion²³—biological activities characteristic of

the wild-type protein. From the predicted amino acid sequence of the mutant HA there would be no reason to suppose that any alteration had occurred in the structure or function of either its binding site¹⁷ or of its fusion peptide^{17,40}. The failure of the mutant form of HA to display these functions is almost certainly a consequence of its never being an integral part of the plasma membrane. Thus, the protein cannot form the necessary bridge between the lipid bilayer of the infected cell and that of the erythrocyte or neighbouring cell. Finally, the secreted HA has no haemagglutinating activity because, lacking the C-terminal hydrophobic region, the protein cannot aggregate to form rosette structures that bind erythrocytes polyvalently.

By contrast to the wild-type and anchor-minus proteins, the signal-minus HA is expressed at low levels from the recombinant virus vector (10⁶ molecules per cell at 60 h post-infection). Further studies are underway to determine in what way the alterations in sequence at the 5' end of the gene may be responsible for the instability of the mutant mRNA. Despite the low levels of expression, our results indicate that the signal-minus protein is an intracellular form of HA probably synthesized on free polyribosomes in the cytoplasm. Cells expressing this protein do not display erythrocyte binding or cell fusion activity—results which are consistent both with the absence of carbohydrate from the molecule⁴¹ and with its failure to reach the cell surface.

The most straightforward explanations of the two mutants' properties are as follows. In the absence of the N-terminal signal sequence there can be no interaction of the nascent HA polypeptide with receptors on the endoplasmic reticulum membrane^{42,43}; translocation of the growing polypeptide across the lipid bilayer therefore cannot occur. Consequently, translation of the mutant mRNA takes place in the cytoplasm and the nascent protein never comes into contact with the glycosylating enzymes that reside in the lumen of the endoplasmic reticulum³⁴. On the other hand, the removal of the C-terminal hydrophobic sequence results in a loss of anchoring function, so that the nascent polypeptide, instead of remaining attached to the luminal face of the rough endoplasmic reticulum, passes completely through the membrane. Once free in the lumen, the mutant form of HA is treated by the cell as if it were an authentic secretory protein, and it is discharged into the medium. It therefore follows that apart from the signal sequences that facilitate their transport into the endoplasmic reticulum, secretory proteins as a class do not contain additional specific amino acid sequences that confer on them the ability to be secreted from the cell.

It is generally assumed that all secretory and integral membrane proteins are transported in vesicles from their site of synthesis on the endoplasmic reticulum via the Golgi apparatus to the plasma membrane. However, it is not known whether the two types of protein are sequestered into different vesicles or whether they follow exactly the same intracellular route. Although the number of proteins studied remains small, it appears that in general secreted proteins move more slowly than membrane proteins to the cell surface and that most, if not all, of this difference in rate arises in the part of the intracellular pathway between the endoplasmic reticulum and the Golgi apparatus⁴⁴. Our results indicate that the transport of the two forms of HA also conforms to this pattern. Thus, by contrast to the membrane-bound wild-type protein which is glycosylated rapidly and relatively synchronously, the population of secretory molecules becomes terminally glycosylated over a very protracted period. It is possible that this difference in some measure reflects the relative efficiency with which membrane-bound proteins and luminal proteins are sequestered into the transport vesicles that travel from the rough endoplasmic reticulum to the Golgi apparatus. Even though the rate of glycosylation of anchor-minus HA is comparatively slow, the composition of the oligosaccharides attached to the wild-type and anchor-minus forms of the protein are not significantly different. It therefore appears that both the secreted and membrane-bound forms of the protein undergo the same sequence of modification reactions during their passage through the Golgi apparatus, and that the signals governing the pattern of glycosylation of HA cannot reside in the 38 amino acids that normally comprise the C-terminus of the protein.

While the mutant forms of HA have been artificially created *in vitro* by deleting portions of the gene, natural counterparts

of these variants are found in eukaryotic cells. *Saccharomyces cerevisiae* produces, from a single gene, distinct mRNA species that code for secreted and cytoplasmic forms of invertase⁴⁵. The secreted enzyme, which is glycosylated, is derived from a precursor that carries a 19-residue N-terminal signal sequence. By contrast, the cytoplasmic form of the enzyme is neither glycosylated nor derived from a precursor. In murine lymphocytes a single gene codes for both membrane-bound and secreted forms of immunoglobulin heavy chains^{10,46,47}. Developmentally regulated alternative pathways of RNA processing give rise to either of two mRNAs, one of which encodes the secreted protein with a hydrophilic C-terminus, while the second encodes in addition a hydrophobic extension that anchors the protein in the membrane.

Finally, the studies with HA can be extended in several ways. For example, the functions of the hydrophobic regions can be analysed in fine detail by creating smaller deletions, insertions and point mutations at particular sites within the cloned HA gene. It will also be possible to construct genes coding for chimaeric proteins, in which the various functional domains of one membrane protein are exchanged for those of another. In this way it can be determined if signal and anchor sequences can be interchanged between different membrane proteins. Meanwhile, the work already accomplished is an illustration of what may become a general method for obtaining with ease large quantities of purified eukaryotic membrane antigens for experimental analysis or vaccine production. (After this manuscript was submitted a paper was published by Sveda *et al.*⁴⁸ on a similar topic.)

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Asymmetric ligand binding by haemoglobin

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The asymmetry observed in the titration curve of haemoglobin with oxygen is possible only if the α - α and β - β subunit interactions change by different amounts on oxygenation. This necessary difference justifies the $\alpha_2\beta_2$ structure on functional grounds and its magnitude shows that haemoglobin function results from reversible oxygen binding to the β subunit.

THE binding of oxygen to haemoglobin is generally presented as a plot of the logarithm of the oxygen concentration against the number of ligands bound per protein molecule (Bjerrum plot). Allen, Guthe and Wyman¹ obtained as the condition of

symmetry of this plot about the midpoint a relation between the four dissociation constants: $K_1K_4 = K_2K_3$, and observed that it required symmetric haem-haem interactions. They believed that departures from symmetry were probably

Table 1 Relative oxygen dissociation constants, asymmetries and computed binding constraints (kcal mol⁻¹) of haemoglobin A

Condition	K_1	K_2	K_3	K_4	Λ	$\{\alpha_i\beta_j\}$	$\{\alpha_i\beta_i\}$	$\{\beta\beta\}$
Stripped ⁶	55.2	14.7	5.82	1	+0.44	1.21	0.96	0.57
HB-ATP ⁶	345	124	73	1	+3.26	2.00	0.68	2.01
Hb-DPG ⁶	545	118	218	1	+3.85	3.24	0.38	2.32
Hb-IHP ⁵	127	56	224	1	+4.59	3.24	-0.41	2.72
Hb in 0.1 M phosphate ⁷	542	106	153	1	+3.40	3.05	0.55	2.09

Computation of constraints made under the assumptions: $\delta I = 0$, $\{\alpha\alpha\} = 0$, first order constraints (equation (14)). The later work of Tyuma *et al.*³ agrees well with the data: stripped Hb, $\Lambda = 0.87 \pm 0.35$; DPG-Hb, $\Lambda = 4.99 \pm 0.45$; IHP-Hb, $\Lambda = 4.14 \pm 0.45$.

artificial, but Roughton *et al.*² showed that there was significant asymmetry in the titration curve and more recently Tyuma *et al.*³ saw a very marked increase in asymmetry on addition of phosphoric effectors to stripped haemoglobin. It is the purpose of this article to relate the asymmetry of titration curves for any number of binding sites to the asymmetry in the functions describing the ligand distribution among the protein molecules, and to show that in haemoglobin these asymmetries necessarily require a considerable difference in the change in interaction energies in the α - α and β - β boundaries upon oxygenation.

Symmetry of ligand distribution and of titration curve

When a protein binds several ligands at sites of identical affinity the distribution of ligands among the protein molecules is the normal Bernoulli distribution⁴. If on average $\bar{n}$ ligands are bound per protein molecule at N possible sites, the fraction of protein molecules binding J ligands is

$$f_J(\bar{n}) = \binom{N}{J} (\bar{n}/N)^J (1 - (\bar{n}/N))^{N-J} \quad (1)$$

where $\bar{n}/N$ is the average probability of occupancy of a site. The plots of f_J against $\bar{n}$, which specify the ligand distribution as $\bar{n}$ increases from 0 to N are, in the case of the normal distribution, symmetric about $\bar{n} = N/2$ in the sense indicated by the equation

$$f_J(\bar{n}) = f_{N-J}(N - \bar{n}) \quad (2)$$

The symmetry of the distributions that are obtained when the

binding sites have arbitrary, rather than equal affinities for the ligand may be readily derived. The dependence of $\bar{n}$ upon the concentration of free ligand $[X]$, in equilibrium with the bound species^{4,5} is described by an equation of the form

$$\bar{n} = \sum_{J=0}^N J \phi_J([X]) / \sum_{J=0}^N \phi_J([X]) \quad (3)$$

$$\phi_J([X]) = \binom{N}{J} \frac{[X]^J}{K_1 K_2 \dots K_J} \quad (4)$$

K_J ($1 \leq J \leq N$) is the intrinsic dissociation constant of the PX_J complex into the components X and PX_{J-1} . The dependence of the distribution of ligands upon $[X]$ is given by

$$f_J(\bar{n}) = \phi_J([X]) / \sum_{J=0}^N \phi_J([X]) \quad (5)$$

Let

$$\sum_{J=0}^N \phi_J([X_{\bar{n}}]) = P_{\bar{n}}; \quad K_1 \dots K_J = a_J \quad (6)$$

where $[X_{\bar{n}}]$ corresponds to an equilibrium with $\bar{n}$ bound ligands. Then equation (2) gives

$$\frac{[X_{\bar{n}}]^J}{[X_{N-\bar{n}}]^{N-J}} \frac{a_{N-J}}{a_J} = \frac{P_{\bar{n}}}{P_{N-\bar{n}}} = \frac{[X_{\bar{n}}]^{J+1}}{[X_{N-\bar{n}}]^{N-J-1}} \frac{a_{N-J-1}}{a_{J+1}} \quad (7)$$

which reduces to

$$[X_{\bar{n}}][X_{N-\bar{n}}] = K_{N-J} K_{J+1} \quad (8)$$

Equation (8) shows that a plot of $\log [X]$ against $\bar{n}$ symmetric about $N/2$ implies a ligand distribution which is also symmetric about $N/2$. Explicit knowledge of the dissociation constants is unnecessary to demonstrate the symmetry of ligand distribution, the constancy of the product $[X_{\bar{n}}][X_{N-\bar{n}}]$ being alone required. Further, equation (8) implies that if the ligand distribution is symmetric about $N/2$, products of pairs of the dissociation constants are equal. The ligand distribution for $N = 2$ is always symmetric. For $N = 3$ equation (9) gives

$$K_3 K_1 = K_2^2 \quad (9)$$

and for $N = 4$

$$K_2 K_3 = K_1 K_4 \quad (10)$$

Figure 1 shows plots of $\log [X_{\bar{n}}][X_{4-\bar{n}}]$ against $\bar{n}$ for two systems, with $N = 4$ and $K_4/K_1 = 43$. $K_3 K_2/K_1 K_4$ has been made equal to 4.59 and 1/4.59. Starting from the same point at $\bar{n} = 0$ the plots diverge towards values respectively larger and smaller than the initial one and tend to a constant value as $\bar{n}$ approaches 2. The asymmetry is seen to be of opposite nature according to whether $K_4 K_1$ is smaller or larger than $K_2 K_3$. To indicate the character and magnitude of the binding asymmetry I shall use the quantity

$$\Lambda = \ln (K_2 K_3 / K_1 K_4) \quad (11)$$

The choice of the logarithm ensures that reciprocal values of the ratio of products of constants are represented by the same figure with opposite sign and reserves $\Lambda = 0$ for the symmetric case. As shown in the figure the logarithmic increment

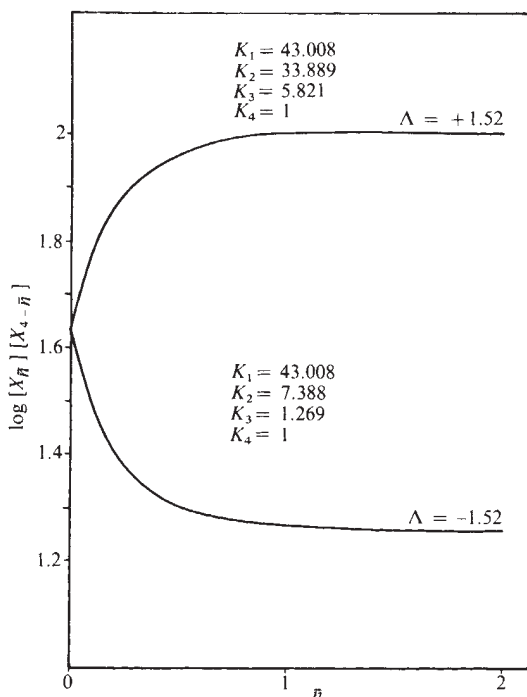


Fig. 1 A plot to demonstrate the opposite effects of negative and positive asymmetry of binding upon the titration curve.

for the positive asymmetry is the same as the logarithmic decrement for the negative one. The significance of positive versus negative asymmetry is disclosed in Fig. 2 which shows plots of f_j against $\bar{n}$ for the systems of Fig. 1. It is a general property of the distributions specified by equation (3) that f_j reaches a maximum at $\bar{n} = J$, whether the distribution is symmetric or otherwise. It is apparent from the figure that in the positive asymmetry the distribution is skewed so that $f_1(1) > f_3(3)$, while for the negative asymmetry the opposite obtains: $f_1(1) < f_3(3)$. Both distributions shown on Fig. 2 have equal Hill coefficients at $\bar{n} = 2$ of ~ 2.4 . The result of the skewing towards f_1 in positive asymmetry is that the cooperative character of the binding is retained at values of $\bar{n}$ closer to 4 in comparison with the negative asymmetry, which exhibits the larger cooperativity at $\bar{n} < 1$. Consequently a smaller decrease in ligand concentration is required to produce the same unloading of X from the protein with positive asymmetry, at values of $\bar{n}$ close to 4. This is clearly shown in the Bjerrum plots of Fig. 3. On this basis alone one expects haemoglobin, which physiologically operates within the saturation range of 0.75 and 0.99+ to exhibit positive asymmetry. If the function of haemoglobin was to scavenge oxygen rather than to work as an almost saturated carrier a negative binding asymmetry would be beneficial to its function.

Origin of the asymmetric ligation

"When effects are asymmetric, the asymmetry should be apparent in their causes", said Pierre Curie in 1893⁶. To explain the origin of the asymmetry we take as an example a tetramer of the type $\alpha_2\beta_2$. This will permit direct application to haemoglobin, the only protein aggregate for which structure and properties are known in sufficient detail to permit this kind of analysis. In an $\alpha_2\beta_2$ tetramer there are six possible subunit interactions: of these the interactions at $\alpha_1\beta_1$ and $\alpha_2\beta_2$ and those at $\alpha_1\beta_2$ and $\alpha_2\beta_1$ respectively equal because of the structural symmetry, so that there are only four different interactions that we shall call $\alpha_i\beta_i$, $\alpha_i\beta_j$, $\alpha\alpha$ and $\beta\beta$. To lighten the notation we designate as $\bar{\alpha}$ and $\bar{\beta}$ the Gibbs free energy changes on binding of dioxygen to the subunits, in the absence of changes in subunit interactions, and we designate as $\{\alpha_i\beta_i\}$, $\{\alpha_i\beta_j\}$, $\{\alpha\alpha\}$, $\{\beta\beta\}$ the difference in free energy of the intersubunit binding between unliganded and fully liganded haemoglobin. These differences have the opposite sign to the free energies of binding, if they are binding constraints, and a wealth of observations indicate that in haemoglobin the subunit interactions operate as binding constraints. To compute the effects that may be expected upon ligation it is necessary to specify how the constraints disappear as successive units are liganded and to this purpose we distinguish two possibilities: a first order constraint, which disappears when a subunit at either side of the boundary is liganded, and a second order constraint, which disappears only when the two subunits across the boundary are liganded. Constraints of the third or fourth order may indeed exist but they must have a very secondary importance in relation of those of first or second order and we neglect them in our treatment. The insignificance of constraints of order intermediate between the first and the second will be made clear shortly.

Computation of the binding asymmetry

The Gibbs free-energy levels $G_0, G_1, \dots, G_4$, corresponding to the species P, PX, $\dots$, PX_4 respectively, are related to the dissociation constants appearing in equation (4) by the equation:

$$\ln K_j = \frac{1}{RT} (G_j - G_{j-1}) \quad 1 \leq j \leq 4 \quad (12)$$

If the free-energies are expressed in RT units, as we shall do in the following, RT disappears in the last and all subsequent equations involving the G values and the binding asymmetry expressed as a function of them is, from (11) and (12)

$$\Lambda = G_0 - 2G_1 + 2G_3 - G_4 \quad (13)$$

which shows that computation of Λ does not require the explicit

value of G_2 . The contribution to the chemical potentials resulting from the interaction of two binding sites is always expressible as a free-energy coupling between them⁷. Binding constraints are antagonistic free energy couplings between the binding of one subunit by another and the binding of either to the ligand. The constraints present when possible sets of sites are liganded are recorded below, where $S = \{\alpha_i\beta_i\} + \{\alpha_i\beta_j\}$

Ligands bound	C (sites filled)	1st order value	2nd order value
0	C_0	$2S + \{\alpha\alpha\} + \{\beta\beta\}$	$2S + \{\alpha\alpha\} + \{\beta\beta\}$
1(α)	C_α	$S + \{\beta\beta\}$	C_0
1(β)	C_β	$S + \{\alpha\alpha\}$	C_0
2($\alpha\alpha$)	$C_{\alpha\alpha}$	$\{\beta\beta\}$	$C_0 - \{\alpha\alpha\}$
2($\beta\beta$)	$C_{\beta\beta}$	$\{\alpha\alpha\}$	$C_0 - \{\beta\beta\}$
2($\alpha_i\beta_i$)	$C_{\alpha_i\beta_i}$	$\{\alpha_i\beta_i\}$	$C_0 - \{\alpha_i\beta_i\}$
2($\alpha_i\beta_j$)	$C_{\alpha_i\beta_j}$	$\{\alpha_i\beta_j\}$	$C_0 - \{\alpha_i\beta_j\}$
3($\alpha\alpha\beta$)	$C_{\alpha\alpha\beta}$	0	$S + \{\beta\beta\}$
3($\alpha\beta\beta$)	$C_{\alpha\beta\beta}$	0	$S + \{\alpha\alpha\}$
4	C_4	0	0

The contribution to the Gibbs levels from each ligand configuration of the table amounts to

$$G(\alpha, \beta) = C(\alpha, \beta) + k\bar{\alpha} + m\bar{\beta} \quad (15)$$

where $0 \leq k, m \leq 2$; $k + m = j$.

Some of the Gibbs levels are manifolds and the contributions of equation (15) must be weighted⁴ to obtain the average value. For G_1 and G_3 the weights are:

$$f_\alpha = \exp(C_\alpha + \bar{\alpha}) / (\exp(C_\alpha + \bar{\alpha}) + \exp(C_\beta + \bar{\beta})) = 1 - f_\beta \quad (16)$$

$$f_{\alpha\alpha\beta} = \exp(C_\alpha + 2\bar{\alpha} + \bar{\beta}) / (\exp(C_\alpha + 2\bar{\alpha} + \bar{\beta}) + \exp(C_\beta + \bar{\alpha} + 2\bar{\beta})) = 1 - f_{\alpha\beta\beta} \quad (17)$$

With the constraints of (14), (13) gives, for first order constraints

$$\Lambda_1 = 2(\bar{\alpha} - \bar{\beta})[f_{\alpha\alpha\beta} - f_\alpha] - [\{\alpha\alpha\} - \{\beta\beta\}](2f_\alpha - 1)$$

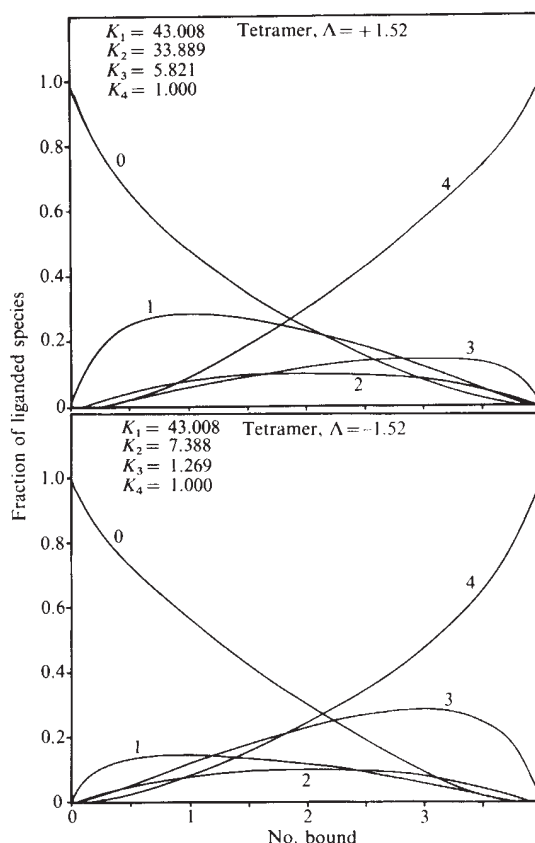


Fig. 2 Skewing in opposite senses of the distribution of ligands for the tetramers of Fig. 1. Numbers on the curves are J values.

and for second order constraints,

$$\Lambda_2 = 2(\bar{\alpha} - \bar{\beta})[f_{\alpha\beta} - f_{\alpha}] + [\{\alpha\alpha\} - \{\beta\beta\}](2f_{\alpha\beta} - 1) \quad (18)$$

and with the substitutions $\delta I = \bar{\alpha} - \bar{\beta}$, $\delta D = \{\alpha\alpha\} - \{\beta\beta\}$ and the f values of equations (16) and (17)

$$\Lambda_1 = \frac{(2\delta I + \delta D)e^{\delta I}(e^{\delta D} - 1) + \delta D(e^{2\delta I + \delta D} - 1)}{(1 + e^{\delta I})(1 + e^{\delta I + \delta D})} = -\Lambda_2 \quad (19)$$

From the last equation,

$$\Lambda_1 = \Lambda_2 = 0 \quad \text{if } \delta D = 0 \quad (20)$$

Therefore, it is a necessary condition for asymmetric ligation, that $\delta D \neq 0$; the 'diagonal' intersubunit couplings $\{\alpha\alpha\}$ and $\{\beta\beta\}$, therefore, must be different. The difference in affinity of the α and β subunits cannot by itself give rise to asymmetry of ligation. If $\delta I = 0$

$$\Lambda_1 = -\Lambda_2 = \delta D \frac{(e^{\delta D} - 1)}{(e^{\delta D} + 1)} \quad (21)$$

and in this case Λ_1 is always positive and Λ_2 always negative. Figure 4 shows plots of Λ_1 and Λ_2 against δD for fixed values of δI . If δI and δD have the same sign Λ_1 is always positive, Λ_2 always negative. Λ_1 can be negative and Λ_2 can be positive if δI and δD have opposite signs and additionally $|\delta D| < |2\delta I|$, the inversion of sign taking place at the point $\delta D + 2\delta I = 0$. A difference in the intrinsic chemical affinity of the subunits can therefore combine with the difference in the value of the diagonal constraints to modulate the asymmetry and even change its sign. From the plots in Fig. 4, as well as from intuitive considerations, one expects that for constraints of the order of 1.5, that is when ligation at the two sides of the boundary decreases the constraints by equal amounts, all asymmetry should disappear, and this can be easily proved by calculating the value of Λ for this case using the method used to derive the values of Λ_1 and Λ_2 .

Application to haemoglobin

The dissociation constants for the four steps of the reaction with oxygen have been determined by Tyuma and coworkers^{8,9} for stripped haemoglobin and for the complexes of haemoglobin with diphosphoglycerate (DPG), adenosine triphosphate (ATP) and inositolhexaphosphate (IHP). Knowles and Gibson¹⁰ determined them in the course of a detailed study of the equilibrium of oxygen with haemoglobin in 0.1 M phosphate solutions. All these studies, summarized in Table 1, indicate a high degree of positive asymmetry for the solutions in phosphate or phosphoric esters and a still positive, though clearly a much smaller one for stripped haemoglobin. The increase in positive asymmetry on addition of DPG is no small effect that requires the exact determination of the dissociation constants for its

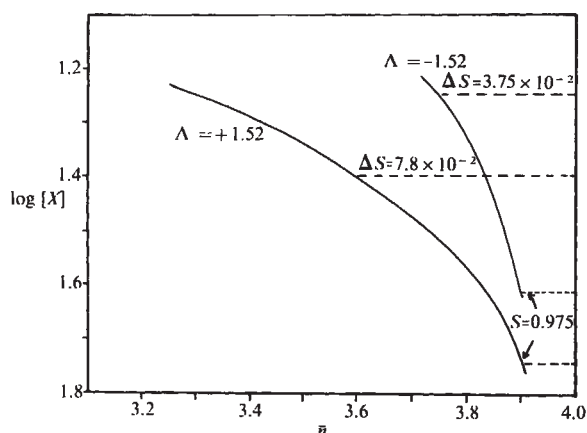


Fig. 3 Differences in the last portion ($\bar{n} > 3.2$) of the Bjerrum plots of the tetramers of Fig. 1, showing the difference in change in saturation (ΔS) for the same increment in free-ligand concentration ($\Delta \log X$).

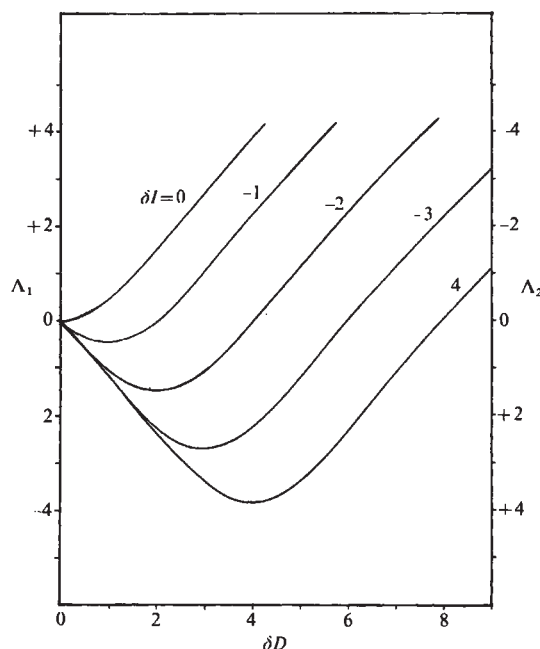


Fig. 4 Plots giving the asymmetry Λ as a function of δD for various values of δI (equation (19)). Λ_1 , scale for first order constraints; Λ_2 , scale for second order constraints.

verification. It is evident in the Bjerrum plots of Fig. 3 of the early paper of Benesch *et al.*¹¹ and in the Hill plots of Fig. 3 of the paper of Tyuma *et al.*⁹. A glance at Fig. 4 indicates that an asymmetry of +3.5 to +4.5 requires a very large difference in the affinities of the α and β chains if the binding constraints are of second order, but a small or null value of δI if the constraints are of the first order. The difference in affinity of the chains is smaller than 1 RT according to Brunori *et al.*¹², and on these grounds it seems safe to conclude that the binding constraints operative in haemoglobin are first order constraints. In calculating the binding constraints from the dissociation constants we found it expedient to set $\delta I = 0$, because the small difference in oxygen affinity of the isolated chains would result in only a minor increase—smaller than 20%—in the value of δD required to satisfy a given asymmetry. Because of the negligible $\alpha\alpha$ boundaries we additionally set $\{\alpha\alpha\} = 0$ or $|\delta D| = \{\beta\beta\}$. With these assumptions the value of the $\{\beta\beta\}$ constraint may be calculated from the asymmetry by means of equation (21) or estimated with acceptable accuracy from the plots of Fig. 4. The total value of the constraints equals the difference between the free energy of binding of the four ligands in the actual system and the free energy of binding to four unconstrained sites:

$$G_4 - G_0 - 4 \ln K_4 = \ln (K_1 K_3 K_2 / K_4^3) = 2S + \{\beta\beta\} \quad (22)$$

The separate symmetric constraints can be determined by fitting the values of G_0 to G_4 to satisfy the experimental quantities. From independent, mainly structural, evidence¹³ we assigned the larger of the two values to the $\alpha_i\beta_i$ boundaries (Table 1). While we dare not make an estimate of the uncertainty of the derived constraints, there is no reason to doubt the validity of the general trends. The $\{\beta\beta\}$ constraint increases systematically in the order: stripped Hb < ATP-Hb < PO₄-Hb < DPG-Hb < IHP-Hb, in agreement with observations that indicate the cross-linking of the isolated β chains by IHP¹⁴. The symmetric constraints are affected in opposite directions by the increase in the $\beta\beta$ constraint. The value of $\alpha_i\beta_i$ increases, but that of $\alpha\beta_i$ decreases to the point that in the IHP complexes this boundary is stabilized, not weakened by ligation. Evidently we are far from the simplicity of two-state models. The recent analysis of mutants of haemoglobin has led Pettigrew *et al.*¹⁵ to conclude, with good evidence, that the $\alpha_i\beta_i$ boundary provides the main constraint to binding and is responsible for the greater part of the cooperativity. This conclusion is supported by the data of

Table 1, which, however, indicates that other constraints, particularly the asymmetric one must play a part. An estimation of the binding asymmetry of mutant haemoglobins seems highly desirable.

I shall end by pointing out those aspects of the function of haemoglobin that can only be adequately explained by reference to asymmetric ligand binding. One is the exclusive correlation of the asymmetry with the diagonal constraint, while the greater part of the affinity and cooperative behavior are determined by the symmetric constraints. This separation of functions in different regions of the molecule entails their independence in physiological control and genetic selection. An α_4 tetramer could not be expected to exhibit differences in the diagonal constraints so that the structure of haemoglobin as an $\alpha_2\beta_2$ tetramer appears now justified on functional grounds. To our knowledge no satisfactory reason for this structure has been previously offered.

In haemoglobin the asymmetry increases in the order: stripped Hb < ATP-Hb < PO₄-Hb < DPG-Hb < IHP-Hb, indicating by itself the importance of this property in the molecular adaptation to lower oxygen pressures. The role of the effector in these cases has been seen as one of reducing the affinity for oxygen but some place must be given to the increase in positive asymmetry as this results in a larger unloading of

oxygen for the same decrease in partial oxygen pressure. In the absence of differences in the intrinsic ligand affinities of the α and β chains preferential binding to either chain can only take place if there is appreciable asymmetric binding. At the values of Λ of stripped haemoglobin there can only be a slight precedence of α over β in the order of binding, but this precedence will rapidly increase with the $\beta\beta$ cross-linking by the phosphoric effectors. At the asymmetry reached with IHP almost complete precedence of α over β may be expected so that the oxygen transport by haemoglobin will result, to a large extent from reversible binding to the β subunit. The work of Chien Ho and his collaborators^{16,17} employing high resolution NMR substantiates this expectation. Considerations of binding asymmetry make clear why haemoglobin is not a suitable model for the regulatory properties of enzymes: haemoglobin works as an easily saturable carrier shuttling between 2/3 and full saturation while enzymes, working more often than not at substrate concentrations that result in modest degrees of ligation, would be expected to benefit from negative binding asymmetry. This could also be at the basis of the often reported partial, or half-of-sites, reactivity¹⁸. The importance of negative binding asymmetry in these and other cases must await a detailed analysis to be carried out with this precise question in mind.

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Activation of a cellular oncogene by DNA rearrangement: possible involvement of an IS-like element

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The cellular oncogene c-mos is rearranged in a mouse myeloma and the tumour mRNA contains transcripts hybridizing with a v-mos probe. The rearranged gene (rc-mos) was cloned in λ phage and shown to transform mouse fibroblasts in transfection assays. rc-mos differs from its progenitor, c-mos, only at the 5' end of the gene, where c-mos sequences have been substituted by a novel cellular DNA fragment. This fragment contains a 159-base pair (bp) insertion sequence (IS)-like element localized immediately 5' to the junction with c-mos. This is the first demonstration in a non-virally-induced tumour of activation of a cellular oncogene by a mechanism possibly involving DNA transposition.

THE nature of the genetic events associated with the malignant transformation of normal cells in natural or induced conditions is little understood. Much of our knowledge of these events has come from studies of the rapidly transforming and slowly transforming retroviruses. It is now well established that the first group of viruses acquired their oncogenic properties by incorporating within their genomes one of a group of cellular *onc* (*c-onc*) genes^{1,2} which are limited in number³, evolutionarily conserved^{4,5} and probably have an essential physiological role in the normal cell and organism. Once incorporated into the viral genome, the *onc* gene (now termed *v-onc*) is controlled by viral signals and expressed in abnormally elevated levels within the infected cell. It is also possible that the viral *onc* gene product differs in subtle but critical ways from its progenitor, the cellular gene. The slowly transforming retroviruses, which do not transduce cellular genes, were shown in some cases^{6,7} to activate transcription of a particular cellular *onc* gene by integration in an adjacent position on the cellular DNA.

The situation is more obscure for the non-virally-induced tumours. By using the DNA-mediated gene transfer approach, it was possible to assay tumour DNAs for their ability to transform mouse fibroblasts in culture. This approach led to the identification of transforming genes in chemically- and radiation-induced tumours^{8–12} and in spontaneous human and mouse tumours^{9,10,13–16}. Recently, three transforming genes from human bladder carcinomas^{17–19} and one human lung carcinoma¹⁷ were identified as the activated forms of the normal human homologue of the viral *onc* genes *Ha-ras* and *Ki-ras*, respectively. Preliminary attempts using restriction enzyme mapping were unable to detect structural differences between the human transforming genes and their normal counterparts^{18,19}. On the other hand, the association of chromosomal aberrations with many murine and human tumours suggested that DNA rearrangements might constitute a general mechanism for tumour induction²⁰, possibly by their effect on cellular oncogenes²¹.

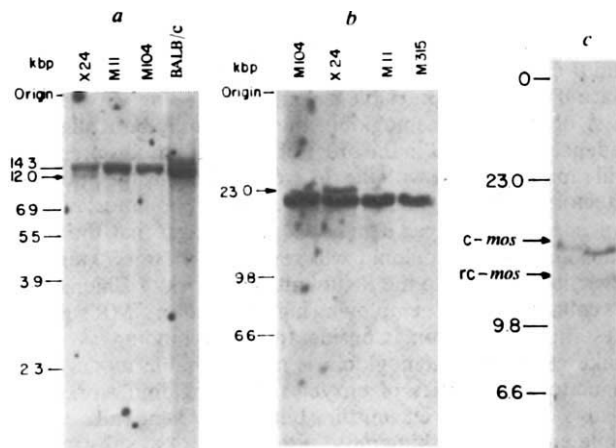


Fig. 1 Analysis of the *c-mos* gene in mouse myeloma DNAs. High molecular weight DNA from mouse myelomas MOPC104E, XRPC24, MPC11 and MOPC315 (referred to in the figure as M104, X24, M11 and M315, respectively) was digested with *Eco*RI (a) or *Bam*HI (b). Aliquots (20 μ g) were electrophoresed on 0.7% agarose gels, blotted onto nitrocellulose filters and hybridized to the *Hind*III-*Bgl*I *v-mos*-specific fragment²³ radio-labelled by nick-translation. After hybridization, the blots were subjected to autoradiography. For molecular cloning, the *c-mos* and *rc-mos* *Eco*RI DNA fragments were enriched from XRPC24 DNA by electrophoresing 500 μ g of digested DNA on 0.5% agarose gel, identifying the location of the two fragments on the gel by hybridization (c) and elution of the DNA by adsorption to glass beads⁵⁰. Arrows indicate the rearranged band.

We undertook the present studies to search in non-virally-induced murine myelomas for the possible activation of another cellular oncogene, *c-mos*. This gene, which is the progenitor of the transforming information in Moloney murine sarcoma virus (M-MuSV)^{22,23}, was characterized extensively by transfection studies²³⁻²⁵ and nucleotide sequencing analysis^{26,27}. These studies established that the cellular information contained within the genome of M-MuSV (denoted *v-mos*), and presumably transduced out of the mouse genome during generation of this virus, consists of a long open reading frame of 369 codons. This sequence is joined in phase to a segment of five codons of viral helper sequence immediately 5' to the *v-mos* region^{26,27}. A 37,000-molecular weight (MW) protein identified within virus-infected cells²⁸ is presumably encoded by the 374-codons sequence and directly triggers cellular malignant transformation. The cellular homologue of *v-mos* (*c-mos*) contains 21 more codons joined in phase 5' to the 369-codon open reading frame described above²⁷. So far, no proteins encoded by this gene have been found in normal cells. Here we show that the *c-mos* gene is biologically activated in a particular mouse myeloma. The activation is due to DNA rearrangement at the 5' end of the cellular gene, involving deletion of *c-mos* information and insertion of a small DNA fragment having structural properties characteristic of an IS element.

***c-mos* gene is rearranged and transcribed in a mouse myeloma**

We screened the DNA of several BALB/c mouse myelomas by Southern blot analysis using the *Hind*III-*Bgl*I *src*-specific fragment of *v-mos*²³ as a probe; one of the tumours—XRPC24—showed an additional hybridizing band detected by this probe (Fig. 1). In the *Eco*RI digest, this additional band was smaller (12.5 kilobase pairs, kbp) than the 14-kbp *c-mos* band, whereas in the *Bam*HI digest the additional band was larger (23 kbp) than the 21-kbp *c-mos* band. These results indicated that the endogenous *c-mos* gene had undergone some rearrangement in the pristine-induced XRPC24 tumour, therefore we will refer to it as *rc-mos*. We attempted to determine whether this DNA rearrangement is associated with enhanced

transcription of the gene. The poly(A)-containing RNA fraction was extracted from the same plasmacytomas and analysed by the Northern technique for *mos*-specific transcripts. Figure 2 shows that the probe detected a transcript of 1.2 kilobase (kb) in the RNA of myeloma XRPC24. No hybridization was observed with RNAs from the other tumours tested. Thus, the appearance of *rc-mos* DNA is correlated with increased transcription of the gene.

To compare *c-mos* and *rc-mos*, we cloned both genes from myeloma XRPC24 DNA digested with *Eco*RI. The 14-kbp and 12.5-kbp *mos* fragments were enriched by preparative agarose gel electrophoresis (see Fig. 1c) and cloned into Charon 4A λ phage vector²⁹. Several clones containing one of the two inserts were purified and analysed in detail.

***c-mos* and *rc-mos* differ at the 5' region**

The two cloned genes were analysed by restriction enzymes; the partial physical maps are shown in Fig. 3. The restriction map of *c-mos* is identical to that published by Vande Woude and co-workers²⁵. *rc-mos* shares with *c-mos* an identical 12-kbp fragment between the *Bgl*I site at 3.1 kbp on the map and the 3' end of the insert. The two clones differ at the region 5' to the *Bgl*I site up to the *Eco*RI site. The sequence of this region in the *rc-mos* clone, determined according to the strategy shown

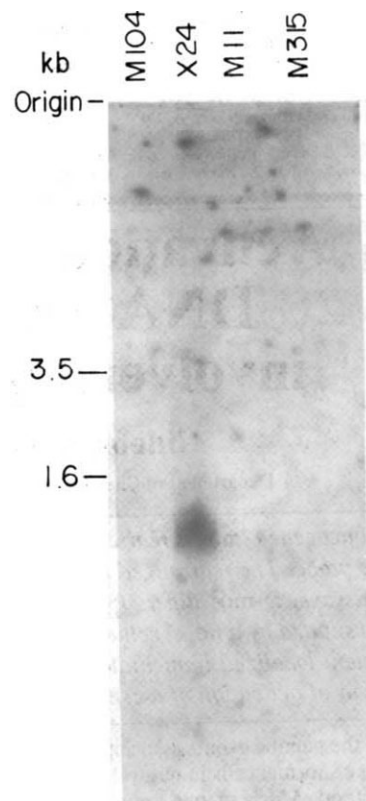


Fig. 2 Analysis of poly(A)⁺ RNA from mouse myeloma for the presence of *c-mos* transcripts. Aliquots (50 μ g) of poly(A)-selected RNA from myelomas MOPC315, MOPC104E, XRPC24 and MPC11 (M315, M104, X24, M11 in the figure) were heated at 60 °C for 10 min in a solution containing 50% formamide, 6% formaldehyde and running buffer (20 mM MOPS pH 7.0, 5 mM NaAc, 1 mM EDTA). The samples were electrophoresed at 100 V for 5 h in 1% agarose gel containing 6% formaldehyde and 1 $\times$ running buffer. The RNA was transferred with 10 $\times$ SSC to nitrocellulose filters, fixed by heating at 80 °C for 2 h and hybridized at 43 °C for 2 days to 2 $\times$ 10⁶ c.p.m. ml⁻¹ of *v-mos* probe in a solution containing 50% formamide, 5 $\times$ SSC, 1 $\times$ Denhardt's mixture, 20 mM sodium phosphate pH 7.0, and 100 μ g ml⁻¹ salmon sperm DNA. After washing at 50 °C with a solution of 0.1 $\times$ SSC, 0.1% SDS, the filters were autoradiographed. Kb, kilobase.

in Fig. 3, is shown in Fig. 4 together with the corresponding published sequence of *c-mos*²⁷.

Clearly, *rc-mos* and *c-mos* are identical 3' to nucleotide 513 and completely divergent 5' of this point. The *c-mos* sequence deleted in the rearranged gene includes about one-quarter of the presumed *c-mos* coding sequence²⁷. The deleted sequence also includes the first 41 codons in the portion of the gene shown to be conserved in both man and mouse³⁰. The inserted novel DNA fragment contains an open reading frame of 28 codons which joins in phase the rest of the *c-mos* open reading frame at nucleotide 513 in the sequence shown in Fig. 4.

Unique structural features of the novel DNA sequences identified in *rc-mos*

Analysis of the *rc-mos* sequence immediately 5' to the junction point (Fig. 4) showed the hexanucleotide CCAACA at positions 507–512. An almost perfect inverted repeat of this sequence—TGATTGG—was found 153 nucleotides upstream at positions 354–360. Further inspection of the sequence indicated that the 159-bp fragment terminated with the inverted repeats, is bound on both sides at positions 347–352 and 513–519 by the hexanucleotide, CACTCC, in a configuration of a direct repeat. As this hexanucleotide is part of the *c-mos* sequence, it must have duplicated during the insertion of the novel 159-bp fragment. Presumably, concomitant or subsequent to this insertion, *c-mos* sequences 5' to the insertion point were deleted. The fragment encompassing the inverted and direct repeats can be drawn in a typical stem-and-loop configuration (see Fig. 5). It is remarkable that the 159-bp fragment ends with the dinucleotide TG...CA as do all eukaryotic movable elements^{31–33}, Mu phage^{34,35} and retroviruses^{36–38}. Finally, the 3'-terminal pentanucleotide of the fragment, CAACA, is identical to the 3' terminus of the *Drosophila* transposable element *copa*³³, yeast *Ty1* mobile element^{31,32}, and spleen necrosis virus DNA³⁶.

Biological activity of the rearranged *c-mos* gene

Previously, it was shown that the molecularly cloned, 14-kbp *c-mos* gene was inactive when assayed for its ability to transform NIH 3T3 cells in transfection experiments²⁵. To demonstrate biological activity of the gene, it was necessary to ligate a viral long terminal repeat (LTR) upstream from the 5' end of the gene²⁵. We asked whether the rearranged *c-mos* gene cloned from myeloma XRPC24 is active by itself when assayed by transfection; a typical experiment is shown in Table 1.

The two clones of the *rc-mos* gene that were tested induced a large number of foci having specific infectivities of 3,100 and 4,900 focus-forming units (FFU) per μ g DNA, respectively (Table 1). In contrast, clones of the 14-kbp DNA fragment containing the normal *c-mos* gene showed negligible or no activity. As a positive control we used two recombinant phages containing integrated Moloney sarcoma viral DNA (E.C., unpublished) which produced 10,200 and 13,200 FFU per μ g DNA (Table 1).

The cells transformed by *rc-mos* DNA were indistinguishable morphologically from those transformed with the viral genome. The transformants consisted of round and spindle-shaped highly refractile cells which grew in multilayers. The foci induced by *rc-mos* DNA could be detected 12 days after transfection in contrast to the 10 days required to detect the viral genome-induced transformants.

Conclusions

Here we have described a genetic event which led to the activation of a cellular *onc* gene in a non-virally-induced tumour. The normal *c-mos* gene has undergone DNA rearrangements in the murine myeloma XRPC24 and has become highly potent in transforming mouse fibroblast monolayers. The biological activation was associated with the appearance of *c-mos* gene transcripts in the tumour tissue. This is all the more

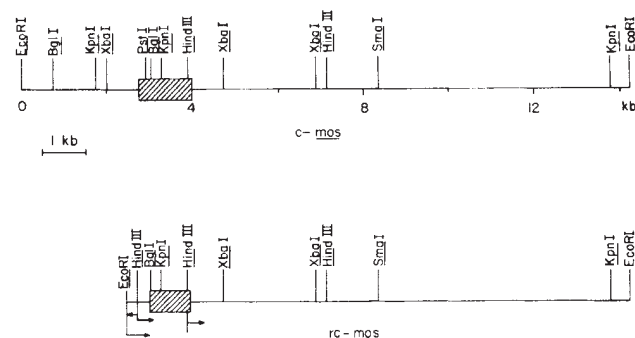


Fig. 3 Physical map of *rc-mos* and *c-mos*. The arrows indicate the strategy for DNA sequencing of *rc-mos*.

notable as, unlike many cellular *onc* genes which are expressed in various normal and malignant cells^{39–42}, *c-mos* gene transcripts have not been detected previously in all cells examined⁴³. The rearrangement of the gene was twofold: first, all sequences 5' to position 513 (Fig. 4), including 263 nucleotides of the presumed coding sequence, were deleted; second, this information was substituted by a novel cellular DNA fragment. Within this fragment, the 3'-terminal 159-nucleotide sequence demonstrated several features (see Fig. 5) in common with bacterial IS elements^{44,45}, Mu phage^{34,35}, eukaryotic transposable elements^{31–33} and retroviruses^{36–38}: (1) Like all these elements, the sequence ends in a terminal inverted repeat. (2) The 3'-terminal two nucleotides, CA, and the 5'-terminal TG are common to all eukaryotic cellular movable elements, Mu phage and retroviruses. (3) The 3'-terminal five nucleotides, CAACA, are shared with the terminal sequence of yeast *Ty1* and *Drosophila copia* elements as well as with spleen necrosis virus DNA. (4) The sequence is bound on both sides by a direct repeat derived from *c-mos* DNA at the junction site. Similar short sequence duplications of the integration site are characteristic of all elements described above. Because of the small size of the sequence found here—159 bp—and the fact that it does not consist of two long terminal direct repeats, we consider the element to resemble most strongly bacterial IS elements or the *Ty1*-related solo δ units found in yeast⁴⁶. Inspection of the sequence reveals neither transcription initiation and termination signals characteristic of retroviral long terminal repeats, nor any other homology to these viral sequences. By using hybridization methods, we detected no sequence homology between the DNA segment inserted 5' to *c-mos* and Moloney murine leukaemia viral DNA. Moreover, our preliminary results indicate that although sequences within this DNA segment are present in normal mouse cell DNA in multiple copies, unlike endogenous murine retroviral sequences⁴⁷, they distribute in a highly conserved pattern in different mouse strains.

Table 1 Biological activity of molecularly cloned *c-mos* and *rc-mos* DNAs

Clone	Insert DNA (ng)	No. of foci	Specific infectivity (FFU per pmol DNA)
λ -MSV 26	50	150	10,200
λ -MSV 3-3	100	220	13,200
λ - <i>c-mos</i> 1A	125	0	67
λ - <i>c-mos</i> 1B	125	1	<67
λ - <i>rc-mos</i> 2B	100	43	3,100
λ - <i>rc-mos</i> 2F	100	68	4,900

Intact recombinant phage DNA was transfected into NIH 3T3 fibroblasts by the calcium phosphate method⁴⁹. The cultures were kept in medium containing 5% fetal calf serum and were scored for focus formation 2 weeks after transfection. λ -MSV 26 and λ -MSV 3-3 represent M-MuSV molecules integrated within normal rat kidney cell DNA and molecularly cloned in λ Wes B vector. λ -*c-mos* and λ -*rc-mos* are molecular clones of the *c-mos* and *rc-mos* genes, respectively, obtained from XRPC24 myeloma DNA.

rc mos	TCTTCTTAA CAGTCTGCT TTACGGGAA CCITTIATTA CCGTGACCC GCAGTTCG GTTCTGGAA TGAGGGATC TTCCTTGGC CCGTCCCG	90
rc mos	AGTTTTCTT GTATTTTTT COTCCCGA TTTTCTCT GTCTGATT TTCTGTCG CGGATTTCAC GCACCAATT GTTATTAGA CGGTTCTC	180
rc mos	ACGACCGGC CAGGAAGAA CACCACAGA CCAGAATCT TCTGCGGCA HindIII AAGCTTTAT TTCTTACAT CTTCAGGAG CCAGGGTCG AGGAAGCAA	270
c mos	CCTTGTGGA GTAGTGATA GCACAGATG TGGCTGGTT TTGAGAATC	
rc mos	GAGAGCAAG AAGCAAGAG AGCGAGAAA ACGAAACCC CGTCCCTCT TAAGGAGCA TTCTCTTTC GCTCTGGAG GTGTCACTC CTTGATTGG	360
c mos	K E E G K G T G I E G S N L P C S Q T S L A V P T H F S L V	
c mos	AAGGAAGAA GGGAAAGGA ACTGGGATT GAAGGCAGC AATCTGCCA TGCTCCCAA ACTTCCTG GCTGTCTT ACTCATTC TCCTAGTG	
rc mos	PstI CTGCAGCCA TCGGCCAGT TGAAGTAC GGGGAAGGC AGAGCACAA GTAGTCATA AGATACCTT TGGCAGATC CGCAGATTA TTTGTTTAC	450
c mos	S H V T V P S E G V M P S P L S L C R Y L P R E L S P S V D	
c mos	TCTCATGTG ACTGTCCA TCTAGGGT GTAATGCT TCGCTCTA AGCCTGTG CGCTACCTC CCGCTGAG CTGTGCCA TCGGTGGAC	
rc mos	H L E H R M S A P S C N G E C G A G S Q H T P P R A P G L P	540
c mos	CACCTAGAA CACAGGATG TCAGCGCA TCTGTACC GGGCAATGT GGGCGCGGC TCCCAACAC ACTCTCTCT CGGCTCCC GGACTGCC	
c mos	S R S C S I P L V A P R K A G K L F L G T T P P R A P G L P	
c mos	TCGCGGTCC TGCAGCATT CCTTTGGT GCCCGAGG AAGGCAGG AAGCTCTTC CTGGGGAC	

rc mos Junction

Fig. 4 Sequence of the novel DNA segment inserted into *c-mos*. The fragment encompassing the *EcoRI*-*BglI* region at the 5' end of the *rc-mos* gene (Fig. 3) was sequenced according to Maxam and Gilbert⁵¹. Arrowed lines indicate the position of the direct (solid line) and inverted (broken line) repeats at the borders of the IS-like element. The *c-mos* sequence is shown for comparison, beginning with the first ATG of the open reading frame²⁷. Amino acids translated from the DNA sequence are given in the single letter code. The junction between the novel DNA fragment and the *c-mos* sequence is indicated by a small arrow. The junction²⁶ between the viral helper DNA and the *mos* information within the M-MuSV genome (*v-mos*) is indicated by an open triangle. The sequence numbers are from the *EcoRI* site shown in Fig. 3.

Finally, note that the mechanism by which the DNA rearrangement took place is probably very complex. The removal of *c-mos* sequences 5' to the insertion point suggests a process of insertion followed by deletion.

The mechanism by which the substitution of *c-mos* sequences with the new DNA fragment activated the gene is unclear. It is probable that the increased transcription of the gene is directly related to the change in its biological properties. It is also possible that the alterations in the structure of the putative protein encoded by the *c-mos* gene, had a role in the activation. It is tempting to speculate that the IS-like element inserted in the gene directly triggers the activation. The element could initiate transcription of the *c-mos* gene by introducing a strong promoter⁴⁸. Alternatively, the presence of the element may lead to modifications of the chromatin structure at this site, which could create a putative promoter within the adjacent *c-mos* gene available for transcription. Analysis of the RNA transcripts, together with transfection studies using different DNA fragments, should elucidate the mechanism of activation.

The finding of a cell line synthesizing mRNA (and probably a transforming protein) encoded by the *c-mos* gene, albeit modified, provides the first opportunity of studying *in vivo* transcription of the cellular gene. The ability of the modified gene to transform suggests that the 263 nucleotides at the 5' end of the *c-mos* gene as well as the 5' 67 codons of *v-mos*, are dispensable for the transforming activity.

c-mos represents the third *c-onc* gene after *Ha-ras* and *Ki-ras*¹⁷⁻¹⁹, shown to be activated in a non-virally-induced

tumour. The modification here involved rearrangement at the 5' end of the gene, including insertion of a 159-bp DNA fragment having the structure of an IS element. This is the first example of oncogene activation in a non-virally-induced tumour by a process of DNA transposition. Whether this is unique, or represents a general method of malignant conversion of potentially transforming cellular genes remains to be shown.

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Note added in proof: Rearrangement of cellular oncogenes might not be a very rare event, since we have now found that the *c-myc* gene has undergone rearrangements in several mouse plasmacytomas. In addition, we have recently obtained evidence for the rearrangement of the human *c-mos* gene in a particular Burkitt lymphoma line. In this case the rearranged *c-mos* gene was inserted near an immunoglobulin V_H gene. This rearrangement has probably resulted from the translocation of the end of chromosome 8, where human *c-mos* is located, to chromosome 14, where the human immunoglobulin heavy chain genes reside.

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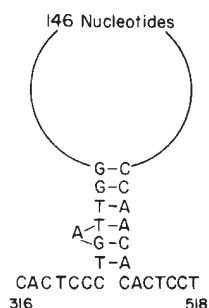


Fig. 5 Schematic representation of the IS-like element present at the *rc-mos* junction. Numbers are as in the nucleotide sequence given in Fig. 4.

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Dramatic growth of mice that develop from eggs microinjected with metallothionein–growth hormone fusion genes

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A DNA fragment containing the promoter of the mouse metallothionein-I gene fused to the structural gene of rat growth hormone was microinjected into the pronuclei of fertilized mouse eggs. Of 21 mice that developed from these eggs, seven carried the fusion gene and six of these grew significantly larger than their littermates. Several of these transgenic mice had extraordinarily high levels of the fusion mRNA in their liver and growth hormone in their serum. This approach has implications for studying the biological effects of growth hormone, as a way to accelerate animal growth, as a model for gigantism, as a means of correcting genetic disease, and as a method of farming valuable gene products.

THE introduction of foreign genes into mammalian embryos could form the basis of a powerful approach for studying gene regulation and the genetic basis of development. Recent studies have clearly established the feasibility of introducing foreign DNA into the mammalian genome by microinjection of DNA molecules of interest into pronuclei of fertilized eggs, followed by insertion of the eggs into the reproductive tracts of foster mothers^{1–7}. The data suggest that the so-called transgenic animals that develop from this procedure integrate the foreign DNA into one of the host chromosomes at an early stage of embryo development. As a result, the foreign DNA is generally transmitted through the germ line^{3,8,9}. In some cases, expression of these foreign genes has also been detected; low levels of rabbit β -globin and viral thymidine kinase (TK) activity were measured in transgenic mice after introducing the respective genes^{6,7}. It is possible to obtain regulated expression of viral TK by fusing the structural gene to the mouse metallothionein-I (MT-I) gene promoter and inducing expression with heavy metals^{2,9}.

The possibility of introducing genes encoding secreted regulatory peptides into animals via this technology has several potential applications. The growth hormone (GH) gene whose protein product has profound developmental effects is particularly convenient for testing this possibility. Based on our success in achieving regulatable expression of herpes TK using the MT-I promoter⁹, we fused this promoter to the rat GH structural gene which has been shown to direct GH synthesis in mouse cells¹⁰. We show here that when this gene construct (MGH) is introduced into eggs it is expressed efficiently, giving rise to greater than normal levels of growth hormone in transgenic mice. The most obvious consequence of this high level of growth hormone expression is gigantism. This success raises several issues of genetic and practical importance.

Generation of animals carrying MGH fusion genes

A fragment of the cloned rat GH gene, from which the 5' regulatory region had been deleted, was fused to the MT-I promoter region, generating the plasmid pMGH (see Fig. 1a). In this construction, the fusion occurs in exon 1, retaining the initiation codon of the GH gene and the 5' regulatory region of the MT-I gene. Thus, the fusion gene is predicted to direct transcription of a mRNA containing 68 bases contributed by MT-I, followed by 1 base contributed by an *Xho*I linker, then the rat GH mRNA sequence starting at nucleotide 7. This construction preserves the AUG initiation codon for GH synthesis located at position 124–126 (Fig. 1c), the four intervening sequences and the poly(A) site of the rat GH gene (Fig. 1b), and 3 kilobases (kb) of downstream chromosomal sequences.

A 5.0-kb fragment extending from the *Bgl*II site of MT-I (–185) to *Bam*HI (see Fig. 1b) was restricted from pMGH, separated from the other fragments on an agarose gel and used for injection into eggs. This linear fragment with heterologous ends was chosen because our recent experience suggests that such fragments integrate into host DNA more efficiently than supercoiled plasmids. Although this fragment contains only 253 base pairs (bp) of MT-I sequence, we have shown previously that this includes sequences essential for heavy metal regulation of the MT-I promoter^{11,12}. The male pronuclei of fertilized eggs were microinjected with 2 μ l containing ~600 copies of this fragment and 170 eggs were inserted into the reproductive tracts of foster mothers; 21 animals developed from these eggs².

When they were weaned, total nucleic acids were extracted from a piece of tail and used for DNA dot hybridization to

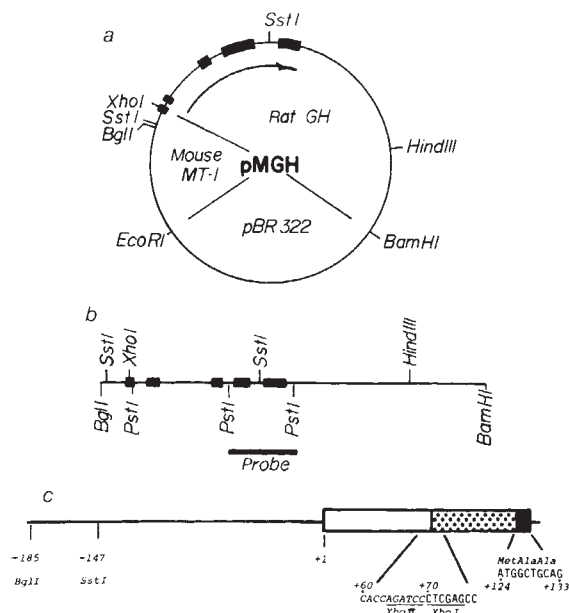


Fig. 1 Construction of the fusion plasmid pMGH. *a*, The unique *Bgl*II site of the MT-1 genomic clone²⁸ was converted to a *Xho*I site by digesting with *Bgl*II, followed by filling in the sticky ends with Klenow fragment of DNA polymerase in the presence of all four dNTPs; then *Xho*I linkers (CCTCGAGG) were ligated to the blunt ends and bacterial strain RR1 was transformed with this DNA. The *Pvu*II site of pBR322 was converted to a *Bam*HI site by a similar procedure, then the 4.1-kb *Xho*I-*Bam*HI fragment containing the MT-1 promoter and pBR was ligated to a 4.8-kb *Xho*I-*Bam*HI fragment containing the rat GH structural gene (see refs 10, 30) to give pMGH (8.9 kb). *b*, The fragment used for injection was a 5.0-kb *Bgl*I-*Bam*HI fragment which was isolated from an agarose gel by the NaClO₄ method²⁹. For genomic Southern blots, a 1.0-kb *Pst*I fragment spanning exons 4 and 5 was isolated and nick-translated⁹ and used as a hybridization probe. *c*, The predicted structure of exon 1 of the fusion gene. The line and open box represent MT-1 untranslated sequences, the stippled box represents GH untranslated sequences and the solid box represents the beginning of the GH coding region (see refs 28 and 30 for complete sequence information).

determine which animals carried MGH sequences. Seven of the animals gave hybridization signals above background (Fig. 2a) and their DNA was analysed further by restriction with *Sst*I and Southern blotting. This analysis showed that all seven had an intact 1.7-kb *Sst*I fragment predicted from the restriction map shown in Fig. 1. All the animals having a prominent 1.7-kb hybridizing band also revealed a 3.3-kb band. This band is predicted from a circularized version of the 5.0-kb *Bgl*I-*Bam*HI fragment. Two of the mice having multiple copies (MGH-10 and -19) gave a 5.0-kb hybridizing fragment when digested with *Hind*III, an enzyme that cuts only once within the injected fragment. Surprisingly, *Bam*HI also generates a 5.0-kb hybridizing fragment in all the DNAs analysed (MGH-10, -14, -16 and -19), suggesting that this restriction site was restored during circularization, whereas *Bgl*I and *Eco*RI (which does not cut within the fragment) gave larger fragments (data not shown). We conclude that all the MGH-positive animals have at least one intact *Bgl*I-*Bam*HI insert and four of the mice have a tandem head-to-tail duplication of this 5.0-kb fragment; however, the details of how and when this fragment circularized and integrated are not discernible. One of the mice, MGH-10, transmitted the MGH genes to half (10 of 19) of its offspring, suggesting that these genes are stably integrated into one of its chromosomes.

Rapid growth of mice carrying MGH genes

At 33 days post-parturition, all of the mice were weaned and maintained on a solid diet supplemented with water containing 5,000 p.p.m. ZnSO₄ (76 mM); their growth rate was recorded

periodically. The dose of Zn was chosen on the basis of experiments which indicated that 5,000 p.p.m. induced almost maximal levels of MT-I mRNA in the liver without impairing the breeding potential of mice maintained on this diet for several months. Figure 3 shows the growth of the seven mice carrying MGH sequences. The littermates without MGH sequences served as convenient controls; the weights for each sex were averaged separately. Six of the mice with MGH sequences showed substantially more weight gain (up to 1.8-fold) than the controls. With the exception of MGH-16, there was a correlation between weight gain and MGH gene dosage (Table 1). The animal carrying the most copies of MGH sequences, MGH-21, died after 7 weeks of rapid growth; other mice have, however, grown larger. Most of the animals were already larger than normal before the Zn diet was instituted. Furthermore, one of the animals (MGH-19) was removed from the Zn diet on day 56 and it continued to grow at an accelerated rate. Thus, the effect of Zn on growth rate is uncertain. Perhaps mice carrying several copies of the MGH gene produce excess GH constitutively without a requirement for heavy metal induction. Thus, the question of Zn-dependent growth will be most easily answered by analysis of offspring of mice such as MGH-16 that contain only a few copies of the MGH gene.

Expression of the MGH gene in the liver

Based on previous studies with mice carrying metallothionein-TK fusion genes, maximal expression of MGH was expected in the liver, intestine and kidney. Evaluation of the tissue specificity of MGH gene expression was initiated by analysis of its activity in liver. A partial hepatectomy on day 56 allowed isolation and quantification of RNA for MGH-specific sequences. Figure 4b shows the results of RNA slot hybridization. The level of MGH mRNA expression in the liver correlated with the growth of the transgenic mice. The largest animals, MGH-2, -19 and -21, contained large amounts of MGH mRNA in the liver, whereas MGH-10, -14 and -16, which showed slower growth rates, contained ~50-fold less MGH RNA, not visible on the exposure shown in Fig. 4. Analysis of samples exposed to base hydrolysis before spotting the nucleic acids on to nitrocellulose (Fig. 4a) or treated with RNases A plus T₁ after hybridization (not shown) established that RNA rather than potentially contaminating DNA was hybridizing to the probe. From these data, we estimate that 30 µg of liver RNA from MGH-2, -19 and -21 contains the same amount of GH mRNA as 13, 25 and 50 ng of poly(A)⁺ rat pituitary RNA,

Table 1 MGH genes, mRNA, GH levels, and growth of transgenic mice

Mouse	Sex	No. of MGH genes per cell	No. of MGH mRNA molecules per cell	Growth hormone (µg ml ⁻¹)	Growth* (g)	Ratio
MGH-2	♀	20	800	57.0	41.2	1.87
MGH-3	♀	1	<50	0.87	22.5	1.02
MGH-10	♂	8	<50	0.28	34.4	1.32
MGH-14	♂	2	<50	0.31	30.6	1.17
MGH-16	♂	2	<50	17.9	36.4	1.40
MGH-19	♂	10	1,500	32.0	44.0	1.69
MGH-21	♀	35	3,000	112.0	39.3	1.78
Female littermates (n = 3)		0	0	0.16	22.0	
				±0.1	±0.8	
Male littermates (n = 11)		0	0	0.15	26.0	
				±0.08	±2	

The number of MGH genes per cell was estimated by DNA dot hybridization and scintillation counting (Fig. 2a). MGH mRNA molecules per cell were estimated by densitometric scanning of the autoradiogram from RNA slot hybridization (Fig. 4) and assuming 2.5×10^5 mRNA molecules per liver cell. GH was measured by RIA as described elsewhere¹⁰.

* Animal weights when 74 days old. The ratio of body weight compared with littermates of the same sex is indicated (see Fig. 3).

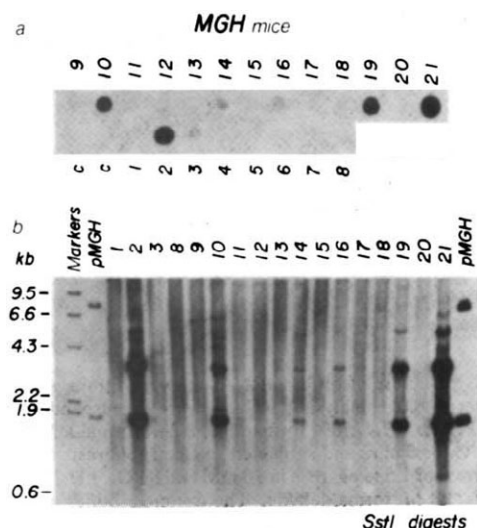


Fig. 2 Analysis of DNA from transgenic mice for MGH sequences. *a*, DNA dot hybridization⁹ was used to detect mice with MGH sequences and quantitate their abundance. A nick-translated *Pst*I probe (see Fig. 1*b*) was used (6 h exposure). Numbers correspond to the MGH mice examined; *c*, controls. *b*, DNA (5 μ g) from MGH mice was restricted with *Sst*I electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridized with the nick-translated *Pst*I probe shown in Fig. 1*b*. pMGH (13 pg, left and 130 pg, right) was included as a hybridization standard. Markers are *Hind*III-cut λ DNA end-labelled with ³²P.

respectively. The RNA standard used contains about 10% GH mRNA on the basis of Rot analysis; therefore, we estimate that there are ~800–3,000 MGH mRNA molecules per liver cell in these transgenic mice (Table 1).

If all processing signals in the fusion gene are correctly recognized, a fusion mRNA 63 nucleotides larger than *bona fide* rat GH mRNA (~1 kb) would be generated^{10,13}. Denaturing gel electrophoresis and RNA Northern blot analysis of the liver RNA from MGH-21 show that its size is similar, as expected, to that of authentic mouse and rat pituitary GH mRNA (Fig. 5*a*). Liver RNA from a control mouse shows no GH-reactive sequences (Fig. 5*a*, lane 1). Because the GH cDNA probe used for the RNA blot analysis recognizes both rat and mouse GH mRNAs, it is necessary to establish that the hybridizing species in the liver is actually the product of the fusion gene and not mouse GH mRNA due to an unexpected activation of the endogenous mouse GH gene. The use of a *Xho*I linker for the construction of the fusion gene generates a sequence that will be uniquely present in MGH mRNA. Thus, the *Xho*I site of pMGH was labelled using [γ -³²P]ATP and polynucleotide kinase, followed by cleavage with *Sst*I (Fig. 1*c*). This 221-nucleotide fragment was gel-purified, denatured, and used as hybridization probe in a single-strand-specific nuclease protection assay. Hybridization to MGH mRNA should generate a 74-base nuclease-resistant fragment while mouse GH mRNA or metallothionein mRNA will be unable to protect the kinased end and should therefore be negative. Figure 5*b*, lane 6 shows that the predicted 74-base fragment is present in liver RNA of mouse MGH-21, but not in normal mouse pituitary RNA (lane 5), control mouse liver RNA (lane 4) or in the liver RNA of MGH-3 (lane 3), an animal showing no accelerated growth (Fig. 3). Thus, it seems that transcription is initiating properly at the MT-I promoter and continuing through the putative termination site of the GH gene, the four GH intervening sequences are being properly spliced and the MGH mRNA is polyadenylated.

Increased growth hormone in sera of MGH mice

The correlation between MGH mRNA levels and growth of the mice suggests that expression of the MGH gene accounts

for the observed biological consequences; this was supported by the three independent RNA analyses above. These results suggest that the circulating levels of GH should be increased. Blood was taken from the transgenic mice and from littermates and assayed for GH by radioimmunoassay (RIA). The GH levels in four of the transgenic mice were 100–800 times greater than levels in control littermates; one mouse had 112 μ g ml⁻¹ of GH in its serum (Table 1). Two of the transgenic mice showing a slow but significantly elevated growth rate had serum GH levels at the high end of the normal range. The lack of physiological regulation and the ectopic production of GH in these animals probably account for their accelerated growth.

Discussion

These data strongly suggest that the altered phenotype of the mice is a direct result of the integration and expression of the metallothionein–growth hormone fusion gene. The elevated level of GH present in some of these mice corresponds to a high level of MGH mRNA in the liver (up to 3,000 molecules per cell). The accumulation of MGH mRNA in the liver is comparable to the endogenous level of MT-I mRNA¹⁴, but is ~100-fold higher than that obtained from metallothionein–TK fusion genes studied previously². This difference is probably the consequence of the intrinsic stability of the GH mRNA relative to TK mRNA; however, differences in transcription rates or processing efficiency due to variations in fusion gene construction are also possible. The high level of MGH gene expression in transgenic mice will greatly facilitate direct comparison of MGH and MT-I mRNA production in different tissues, thus allowing us to determine whether chromosomal location has an important effect on tissue-specific expression of the MT-I promoter.

Growth hormone levels in some of the transgenic mice were as much as 800-fold higher than in normal mice, resulting in animals nearly twice the weight of their unaffected littermates. This greater than normal accumulation of GH undoubtedly reflects both the lack of normal feedback mechanisms and expression of this gene in many large organs including liver, kidney and intestine. The effect of chronic exposure to high levels of GH is well documented¹⁵, resulting in the clinical condition referred to as gigantism. This condition in humans is

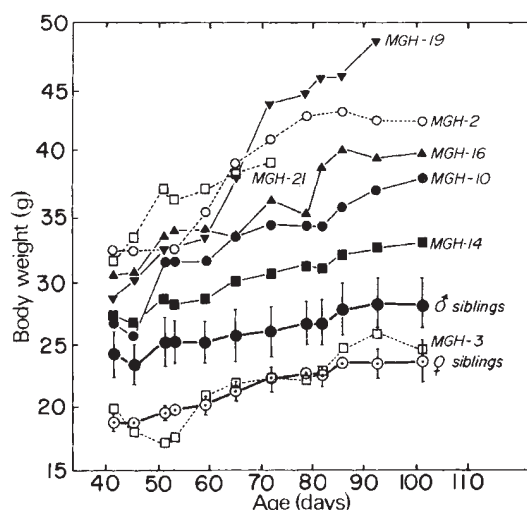


Fig. 3 Growth of MGH mice. Microinjected eggs (SJL×C57) were transferred to oviducts of foster mothers on 14 April; 21 animals were born 3 weeks later. At 33 days old they were weaned and the drinking water was supplemented with 76 mM ZnSO₄. The body weights of the males are shown as solid symbols; the mean weight ($\pm$ s.d.) of 11 siblings not containing MGH sequences is also shown. The female weights are represented by open symbols; means ($\pm$ s.d.) of three siblings are indicated also. MGH-21 died on day 72. A partial hepatectomy was performed on MGH-19 on day 56 and it was taken off Zn thereafter; it was killed on day 100. All mice were taken off Zn after day 83.

usually associated with pituitary adenomas¹⁵ and more rarely with ectopic expression of GH by lung carcinomas^{16,17}.

Some of the diverse effects of GH are mediated directly by the hormone¹⁸⁻²⁰. However, it is generally believed that the major effect of GH is stimulation of somatomedin production in the liver^{18,19}. Somatomedins are insulin-like growth factors that promote proliferation of mesodermal tissues such as muscle, cartilage and bone^{18,19}. The involvement of somatomedins in the GH response provides an explanation for the growth of animals such as MGH-10, 14 and 16 in which the circulating level of GH was only slightly higher than normal. In these animals, GH produced in the liver may be sufficient to stimulate somatomedin production because the local GH concentration is relatively high. Thus, in these animals, GH may mimic the local paracrine function of some hormones²¹. However, the reason for lack of growth of MGH-3 is unclear.

The implicit possibility is to use this technology to stimulate rapid growth of commercially valuable animals. Benefit would presumably accrue from a shorter production time and possibly from increased efficiency of food utilization. The effects of continuously elevated GH levels on the quality of meat will need to be evaluated. By choosing the appropriate GH gene, milk production may be disproportionately increased considering that GH is homologous to prolactin, that GH of some species binds to prolactin receptors, and that exogenously administered GH has been shown to increase milk yield^{22,23}. Having a regulatable promoter may be particularly advantageous for timely expression of GH. Applying these techniques to large animals will be more difficult. Nevertheless, when genes for desired traits can be isolated, this approach should provide a valuable adjunct to traditional breeding methods. Optimizing the conditions for integration and expression of foreign genes

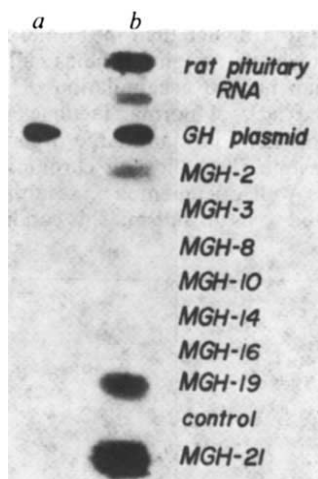


Fig. 4 Slot-blot analysis of liver RNAs from transgenic mice. Mouse liver RNA was purified by homogenizing a liver slice in 1×SET (1% SDS, 5 mM EDTA, 10 mM Tris pH 7.5), 200 µg ml⁻¹ proteinase K (Boehringer) followed by phenol/chloroform extraction, ethanol precipitation, DNase I treatment (Worthington; DPFF), a second phenol/chloroform extraction, then re-precipitation with ethanol and finally resuspension for A₂₆₀ determination. Liver RNA (30 µg) was diluted to 100 µl in 2X SSC. Samples were brought to 0.5 M NaOH or 0.5 M NaCl and incubated at 65 °C for 30 min. 140 µl of 20× SSC and 160 µl of 12.3 M formaldehyde were added, incubated at 65 °C for another 15 min and applied to a nitrocellulose sheet res soaked in 20× SSC through a slot-blot device³¹. The nitrocellulose was baked at 80 °C for 2 h, prehybridized for 5 h and then hybridized with nick-translated ³²P-rGH cDNA³² (5 × 10⁶ c.p.m. in 4 ml at 42 °C overnight), washed in 0.1×SSC at 42 °C, and exposed to X-ray film at -70 °C for 24 h. *a*, Samples were treated with 0.5 M NaOH and neutralized with HCl. *b*, Samples were incubated with an equivalent amount of NaCl. 40 and 20 ng of rat pituitary poly(A)⁺ RNA in the presence of 30 µg of control liver RNA were applied to slots as GH mRNA controls. 1 ng of rGH gene plasmid, also in the presence of 30 µg of control liver RNA, was applied as a base-resistant control.

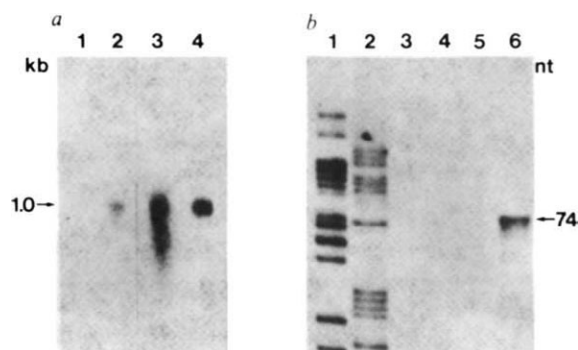


Fig. 5 Structure of MGH mRNAs in liver of transgenic mice. *a*, Northern blot analysis. RNAs were resuspended 10 mM NaH₂PO₄, pH 7.4/50% formamide/2.2 M formaldehyde, heated at 65 °C for 5 min, and subjected to electrophoresis on a slab gel composed of 1.5% agarose in 10 mM NaH₂PO₄, pH 7.4/0.55 mM EDTA/1.1 M formaldehyde. The running buffer was 10 mM NaH₂PO₄, pH 7.4/0.5 M formaldehyde. The gel was stained with acridine orange to identify rRNA markers, photographed, incubated for 90 min with 50 mM NaOH, and then neutralized with two washes of 0.2 M NaOAc, pH 4.3. The RNA was then transferred to diazotized paper and prehybridized as described elsewhere³². Lane 1, total liver RNA (15 µg) from a control littermate; lane 2, total liver RNA (15 µg) from MGH-21; 3, total mouse pituitary RNA; 4, 40 ng of rat pituitary mRNA, poly(A)⁺. Lanes 1 and 2 exposed for 48 h; lanes 3 and 4 exposed for 5 h. *b*, Single-strand-specific nuclease protection assay. 20 µg of RNA were hybridized at 47 °C for 5 h in 40 µl of a solution containing 40 mM PIPES pH 6.4, 0.4 M NaCl, 80% formamide and 50,000 c.p.m. gel-purified 221-bp SsrI-XhoI fragment of pMGH end-labelled at the XhoI site with ³²P (see Fig. 1c). The samples were then diluted with 0.3 ml of 280 mM NaCl, 30 mM NaOAc pH 4.4, 4.5 mM ZnSO₄, 20 µg ml⁻¹ salmon sperm DNA and 150 units of mung bean single-strand-specific nuclease (Collaborative Research) and incubated at 47 °C for 1 h. Samples were ethanol-precipitated, resuspended in 90% formamide containing bromophenol blue and Xylene Cyanol FF, loaded on to an 8% acrylamide-urea sequencing gel, electrophoresed for 1.5 h at 2,000 V, dried and autoradiographed for 7 days at -70 °C with an intensifying screen. Lanes 1 and 2, sequencing ladder used for size standards; 3, MGH-3 RNA; 4, control liver RNA; 5, mouse pituitary RNA; 6, MGH-21 RNA.

in mice should facilitate the eventual application of these techniques to other animals.

Another possibility is the use of this technology either to correct or to mimic certain genetic diseases. There are several inbred dwarf strains of mice, one of which, *little*, lacks GH and is about half normal size when homozygous²⁴. Thus, introduction of the fusion gene described here or a natural GH gene might restore normal growth to these animals. On the other hand, the experiments described above show that gigantism can be created. Once these mice are inbred to give homozygous stocks, they will provide a valuable model system for biochemical studies on the consequences of excess GH production.

Finally, the exceptionally high levels of GH found in the sera of some of these mice raises the possibility of extending this technology to the production of other important polypeptides in farm animals. The concentrations of GH in MGH-21 serum was 10–100-fold higher than that reported for bacterial or mammalian cell cultures that were genetically engineered for GH production^{10,25-27}. This genetic farming concept is comparable to the practice of raising valuable antisera in animals, except that a single injection of a gene into a fertilized egg would substitute for multiple somatic injections; moreover, the expression of that gene is likely to be heritable⁹. This approach may be particularly applicable in those cases where the protein of interest requires special covalent modifications (for example, proteolytic cleavage, glycosylation, or γ-carboxylation) for activity or stability.

Clearly the ability to introduce into mice, and by extrapolation into other animals, functional genes of selected

construction offers wide ranging experimental as well as practical opportunities.

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LETTERS TO NATURE

A millisecond pulsar

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The radio properties of 4C21.53 have been an enigma for many years. First, the object displays interplanetary scintillations (IPS) at 81 MHz, indicating structure smaller than 1 arc s, despite its low galactic latitude (-0.3°)¹. IPS modulation is rare at low latitudes because of interstellar angular broadening. Second, the source has an extremely steep ($\sim \nu^{-2}$) spectrum at decametric wavelengths². This combination of properties suggested that 4C21.53 was either an undetected pulsar or a member of some new class of objects. This puzzle may be resolved by the discovery and related observations of a fast pulsar, 1937+214, with a period of 1.558 ms in the constellation Vulpecula only a few degrees from the direction to the original pulsar, 1919+21. The existence of such a fast pulsar with no evidence either of a new formation event or of present energy losses raises new questions about the origin and evolution of pulsars.

A literature search in 1979 led to the suggestion that the steep-spectrum, IPS source was superposed on a flat-spectrum ($\nu^{-0.1}$) object with a diameter of ~ 60 arc s located to the west of the 4C position by one interferometer lobe (~ 31.6 s). Lobe identification errors in the 4C catalogue can occur in regions of confusion such as the galactic plane.

The superposition of two source components, one very compact with a steep spectrum and the other extended with a flat spectrum, was reminiscent of the radio properties of the Crab nebula and its pulsar in the pre-pulsar era³. The conjecture was made that the compact component in 4C21.53W was a pulsar as yet undetected due to pulse broadening of its radiation over one period by interstellar scattering. However, the IPS measurement placed an upper limit on interstellar scattering which, in turn, placed a limit on the pulse broadening. The conclusion was that only a very short period pulsar, $P \leq 10$ ms, would have been missed in metre wavelength searches owing to this effect. Searches for such a short period pulsar at centi-

metre wavelengths, where pulse broadening would be much reduced, were conducted at Arecibo Observatory and at Owens Valley Radio Observatory in 1979 without success.

After the 1979 pulsar searches, Erickson (personal communication) located a steep-spectrum compact source, 4C21.53E, east of the 4C position by one 4C interferometer lobe ($+31.6$ s). This observation provided evidence against the superposition hypothesis. Furthermore, Very Large Array (VLA) observations at 5 GHz by one of us (D.C.B.) showed that 4C21.53E was a compact double source with separation of 0.8 arc s.

Interest in the extended western object, 4C21.53W, returned when decametric observations at the Clark Lake Radio Observatory showed that both 4C21.53E and 4C21.53W had very steep spectra below 100 MHz (ref. 4). In addition, the Clark Lake observations indicated that the western source showed IPS at 34 MHz. The inferred brightness temperature exceeded $>10^{12}$ K.

Observations of 4C21.53W with the Westerbork Synthesis Radio Telescope (WSRT) at 609 MHz in January 1982

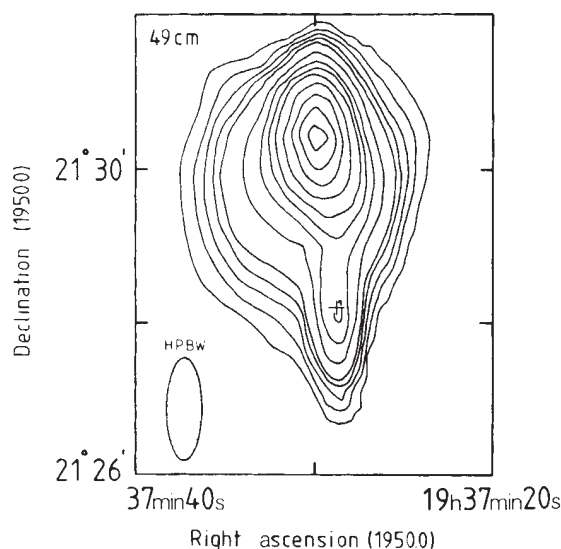


Fig. 1 Image of extended (north) and compact (south, +) components of 4C21.53W from a 12-h synthesis with the WSRT at 608.5 MHz on 15 January 1982. The synthesized beamwidth shown at the lower left is 31.3×80.4 arc s in RA and Dec, respectively.

Table 1 Flux densities of 4C21.53W

Frequency (MHz)	Instrument	Total flux density (Jy)	Compact flux density (Jy)	Compact polarization (%)	Extended flux density (Jy)
430	Arecibo	1.60 ± 0.30	$\sim 0.3^*$	—	$\sim 1.3 \pm 0.30$
609	WSRT	1.15 ± 0.05	0.130	28 ± 2	1.02 ± 0.05
1,380	Arecibo	$0.96^{\dagger} \pm 0.05$	$\sim 0.02^*$	—	0.94 ± 0.05
1,415	WSRT	0.93 ± 0.02	0.017	15 ± 2	0.91 ± 0.02
2,380	Arecibo	$0.90^{\dagger} \pm 0.05$	—	—	0.90 ± 0.05
5,000	Bonn ⁶	0.91 [†]	—	—	0.92

* Estimated from compact source spectral index between 609 and 1,415 MHz.

† Corrected for beam sizes.

confirmed a suspected position discrepancy based on a Culgoora measurement at 80 MHz (ref. 5) and a Bonn measurement at 5,000 MHz (ref. 6) (Fig. 1). We suspected that the Culgoora position was dominated by the steep-spectrum component and that the Bonn position was dominated by the flat-spectrum, extended component. The division of 4C21.53W into two components, evident in Fig. 1, confirmed our suspicion. The southern and northern components were named 4C21.53W(com) or 1937+214 and 4C21.53W(ext) or 1937+215, respectively. A brief observation at the VLA confirmed the position and steep spectrum of 1937+214. A map of 4C21.53W at 1,415 MHz from a 12-h observation with the WSRT in August 1982 clearly resolved the compact and extended source component (Fig. 2). Recent WSRT observations and 1979 total power observations from Arecibo are summarised in Table 1. The spectra are decomposed into compact and extended object contributions. The Bonn measurement⁶ is included for completeness. Erickson's decametric observations of this source are reported elsewhere⁷.

The spectrum of the extended source is approximately that of an H II region, $\nu^{-0.1}$. The H 166 α recombination line was detected at the Arecibo Observatory at the position of the extended source in November 1982. The line temperature is $\sim 1\%$ of the continuum temperature. The velocity of the line is $+2 \text{ km s}^{-1}$ and its FWHM is $\sim 25 \text{ km s}^{-1}$. These properties are comparable with those of an ordinary H II region. The small velocity does not distinguish between kinematic distances of 0 and 8.5 kpc.

The proximity of these two components of 4C21.53W on the sky suggests a possible physical connection. The morphology of the 21-cm continuum maps indicates that the exciting star may be displaced 43 arc s northwards from the centre of the H II region defined by the near-circular, low-level contours. On the other hand, the compact source is displaced 117 arc s southward from the centre. We will return later to the possible connection between these objects.

Interest in further pulsar searches was rekindled following the discovery of a steep-spectrum component in 4C21.53W at decametric wavelengths⁸. Momentum for the search increased when the 609-MHz WSRT map was available. A pulsar search sensitive to periods $>4 \text{ ms}$ was carried out by Boriakoss (personal communication) at the Arecibo Observatory in March 1982 without success. Detection in September 1982 of strong linear polarization at the compact source position in the WSRT maps made a pulsar detection a near certainty.

A new pulsar search was conducted with the 305-m antenna at the Arecibo Observatory on 25 September 1982 at the position of the compact 15 mJy component detected in the 1,400-MHz synthesis observations. Two harmonics of a millisecond periodicity, 1.558 ms, were discovered at the compact source position. The signal was present for only 3 min of a 7 min sample, and was not seen on a following day at either 1,400 or 2,380 MHz.

In November 1982 the pulsar search was intensified at Arecibo. In addition, we planned a search for interstellar scintillation (ISS) based on the possibility that the compact object was small, but not pulsing. Deep ISS modulation was detected

at 1,400 MHz at the position of the compact source (Fig. 3). The amplitude was consistent with the flux density found in synthesis observations. The frequency and time correlation lengths, roughly 2 MHz and 5 min, respectively suggested a relatively small dispersion measure to the object, $<100 \text{ electrons pc cm}^{-3}$. This observation indicated immediately that the compact source 4C21.53W(com) or 1937+214, was extremely small as only pulsars have shown ISS previously. In addition, the modulation bandwidth and time scale were consistent with the single detection in September. On the following day the millisecond pulsar was confirmed.

The waveform of the pulsar contains a main pulse and an interpulse of comparable intensity separated by $\sim 180^\circ$ (Fig. 4). This morphology repeats precisely the main pulse/interpulse morphology of the Crab pulsar. The Crab pulsar has an additional 'precursor' component preceding its main pulse at metre wavelengths. The full widths at half intensity of the pulse components in Fig. 4 are $<125 \mu\text{s}$, or 8% of the period. The pulses are readily detected with the Arecibo telescope at 1,400 and 430 MHz with a fast signal averager and integrations of a few hundred pulses when positioned on a peak of the ISS modulation pattern (Fig. 3). The waveforms at both frequencies are similar.

The first observations have resulted in the following parameters: RA (1950.0) 19h 37min 28.72s, Dec (1950.0) $21^\circ 28' 01.3''$, Barycentric period 0.001 557 807 (JD 244 5282), Dispersion measure 75 electrons pc cm^{-3} . The accuracy of all values is a few parts in the last decimal place. The distance is estimated as 2,500 pc using an average n_e of 0.03 cm^{-3} . There is no evidence for binary motion in timing measurements during the second week in November. Timing observations have been

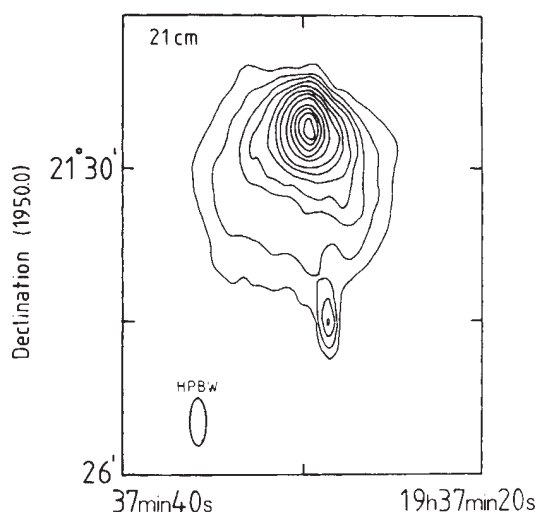


Fig. 2 Image of extended (north) and compact (south) components of 4C21.53W from a 12-h synthesis observation with the WSRT at 1,415 MHz on 8 August 1982. The synthesized beam-width shown at the lower left is 13.3×37.0 in RA and Dec respectively.

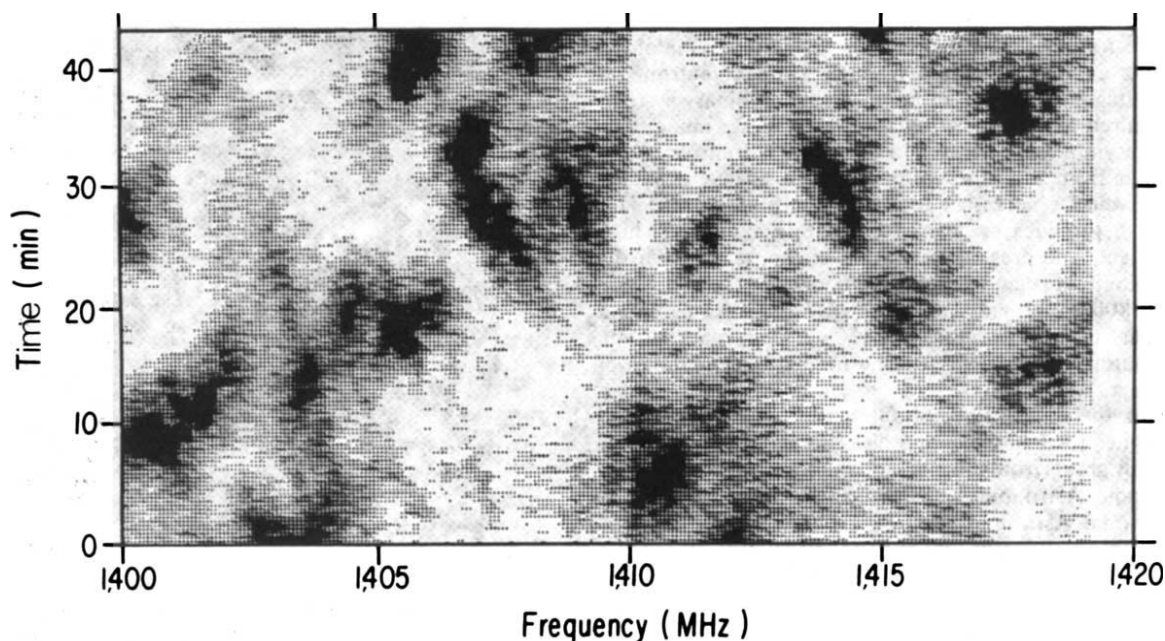


Fig. 3 Interstellar scintillation observation of compact component in 4C21.53W from Arecibo observations on 6 November 1982. Individual spectra were obtained for two polarizations in two 10-MHz bands every 30 s using a 252-channel, 1-bit autocorrelator. Spectra from the two polarization channels were summed and smoothed to a resolution of 100 kHz. The peak intensity in this dynamic spectrum is ~ 50 mJy.

initiated to determine the period derivative at the Arecibo Observatory. A previous estimate of the period derivative⁸, based on a comparison of measured periods in September and November, has not been substantiated by timing data in November. We suspect that the September measurement was corrupted by sampling errors. Analysis of the November observations results in an upper limit to $\dot{P}$ of 10^{-15} s per s.

Our original hypothesis that 4C21.53W was a fast pulsar superposed on an extended synchrotron-emitting nebula was only half correct. Now we are faced with a more profound enigma: the existence of a pulsar rotating near the maximum rate possible for a neutron star with a surprising lack of evidence of energetic activity in the vicinity of the pulsar.

The present rotation rate, 642 Hz, is very near the maximum rate of 2,000 Hz where centrifugal forces balance gravitational forces at the surface of a $1 M_{\odot}$ neutron star with 10 km radius. Matter on the equator would have a velocity of $0.13c$. Changes in the equilibrium figure of the pulsar resulting from energy losses to magnetic dipole and gravitational quadrupole radiation (discussed below) may occur as abrupt starquakes if there is such a close balance between gravitational and centrifugal forces. The balance could be tipped in favour of gravity if the pulsar is denser than $\Omega^2/G \sim 2 \times 10^{14}$ g cm³. The amplitude and frequency of starquakes, observable in pulse arrival time measurements, may be able to distinguish between standard ($1 M_{\odot}$) and high density models for the millisecond pulsar.

The present rotational energy content is 7×10^{51} erg for a neutron star moment of inertia 10^{45} g cm². This energy is comparable to the entire mechanical energy output of a supernova event. A higher density star, as suggested above, could reduce the present energy content if its moment of inertia were smaller than 10^{45} g cm². Both the rapid spin and the large energy content are indicative of a young object as energy losses to magnetic dipole and gravitational quadrupole radiation are strong functions of the rotation rate, Ω^4 and Ω^6 , respectively⁹. The minimum energy loss for this pulsar will be the observed radio emission which amounts to 3×10^{30} erg s⁻¹ assuming a beam solid angle of 1 sr.

The age of this pulsar is puzzling. The maximum spin rate for a neutron star is $\sim 2,000$ Hz. A model for the Crab pulsar by Ostriker and Gunn⁹ predicts a period decay from this rate to 100 Hz in 1 yr due to gravitational quadrupole radiation.

We do not observe such a rapid decay. Furthermore, there is a surprising absence of evidence of any debris from a recent neutron star formation event. Our radio maps show no synchrotron-emitting nebula in the vicinity of the pulsar. Einstein observations place a limit of 1.5×10^6 K for the surface temperature of a neutron star at the pulsar position for the indicated distance of 2 kpc, and exclude the possibility of a synchrotron nebula (D. J. Helfand, personal communication). These X-ray limits do not allow for possible heavy extinction. There is no source at the pulsar position in the COS B catalogue¹⁰. Lick Observatory observations reveal a 20 mag optical star at the position of the pulsar¹¹. In direct analogy to the Crab pulsar, we expect this object will show optical pulsations.

We conclude that despite the large spin and rotational energy, this pulsar is not young. Evidently it has found a way to preserve a large fraction of its original energy. Minimal energy loss requires low values for the perpendicular magnetic dipole moment and the gravitational quadrupole moment. The first binary pulsar, 1913+16, is an example of a rapidly rotating neutron star (17 Hz) with a very low moments based on period derivative measurements. Observations of the spin decay will determine the dominant energy loss mechanism.

Two factors lead us to suggest that the pulsar and the H II region are related: (1) The two objects are in the same area of sky in a region of relatively low radio confusion. (2) The brightest part of the H II region and the pulsar are displaced to opposite sides of the near circular, low-level contours of the H II region.

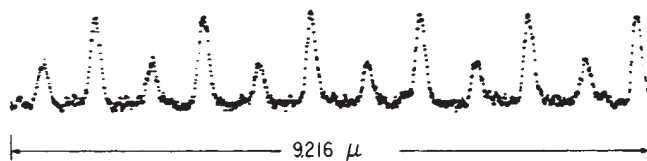


Fig. 4 Waveform of the millisecond pulsar from a signal averager oscilloscope display. Sample spacing is 9μ s. The full trace is roughly six periods, 9,216 μ s. The integration lasted ~ 75 s. The waveform consists of a main pulse and an interpulse separated by nearly 180° of rotational phase. Errors in timing the signal averager and a 20μ s RC time constant are responsible for most of the pulse width.

We propose that the pulsar and the exciting star of the H II region were formerly members of a binary system. One of the components evolved and went through a quiet neutron-star formation stage. Formation of neutron stars in binary systems is also required to explain X-ray and radio pulsar binaries. A large and asymmetric energy and momentum transfer to the neutron star from the other component must have provided the escape velocity necessary to disrupt the binary orbit. This transfer also provides a means for creating a massive, high density object. The present separation of the pulsar from the centre for the H II region suggests an epoch for the disruption event of 7,800 yr ago and a distance of 2 kpc if we assume a typical pulsar transverse velocity of 150 km s^{-1} . Proper motion of the pulsar would be southward in declination of $0.015 \text{ arc s yr}^{-1}$.

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Optical identification of the millisecond pulsar 1937+214

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Backer *et al.*¹ have reported the discovery of a pulsar with the period of 1.558 ms, identified with the source 1937+214 (=4C21.53). The extreme rotational velocity and high energy loss rate of this object make it a very interesting astrophysical phenomenon not only as an important clue for the physics of neutron stars and supernova remnants, but also as a potential strong emitter of gravitational radiation (S. White and J. Arons, personal communication). I present here an optical identification candidate for the millisecond pulsar: it is a 20th mag red object, undetectable on the Palomar Sky Survey prints. Finding charts and offsets from nearby stars are given.

The data were obtained on the night of 14 October 1982 UT, on the Lick Observatory 1-m telescope, in conditions of good seeing (FWHM $\approx 1 \text{ arc s}$). A red-sensitive TI 500 $\times$ 500 thin CCD was used as a detector, together with a sky-suppression red filter, purchased earlier by H. Spinrad. This wide red bandpass has an effective wavelength at about 6,500 Å and the width of $\sim 1,400 \text{ Å}$. Three CCD exposures, one of 300 s and two of 600 s, were obtained. The frames were flatfielded with a 'dome flat', filtered to remove cosmic ray events, and had bad columns masked (standard CCD reduction procedure). The three frames were then offset into coincidence, and digitally stacked together, to increase the signal-to-noise ratio.

Part of the resulting image containing the object is shown in Fig. 1. Figure 2 shows a slightly larger region, enhanced by a new, highly nonlinear algorithm, which will be described elsewhere. The candidate object was found on all three CCD frames, although only the stack is shown here. Figure 3 shows a much larger field, as enlarged from a Palomar print.

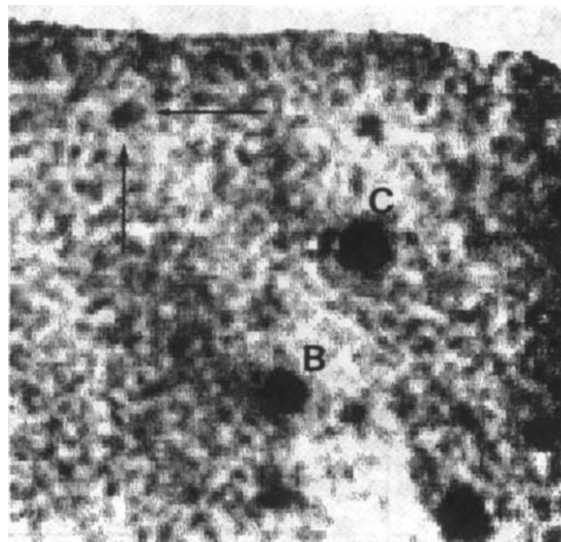


Fig. 1 A section of the CCD stack frame. The pulsar candidate is marked with the arrows. Stars B and C are those from Table 1. The field size is about 50 arc s, the pixel size 0.38 arc s. North is up, east to the left.

I have performed aperture photometry on the digital data of this source. In a pseudo-diaphragm of 6 arc s diameter, I obtain

$$m_r = 20 \pm 1$$

(the error may be an overestimate). The zero-point of the photometry was determined from a standard star, Feige 15, whose exposure was taken immediately after the pulsar field exposures. There is no extended emission associated with this source, down to the limit of these data.

I have also searched for the extended source (H II region?), detected in radio observations, a few arc min to the north from the pulsar, and apparently associated with it. The result of this attempt was negative, but my data (one 600-s exposure) are of insufficient quality to place any interesting limits on the possible existence of extended optical structure there.

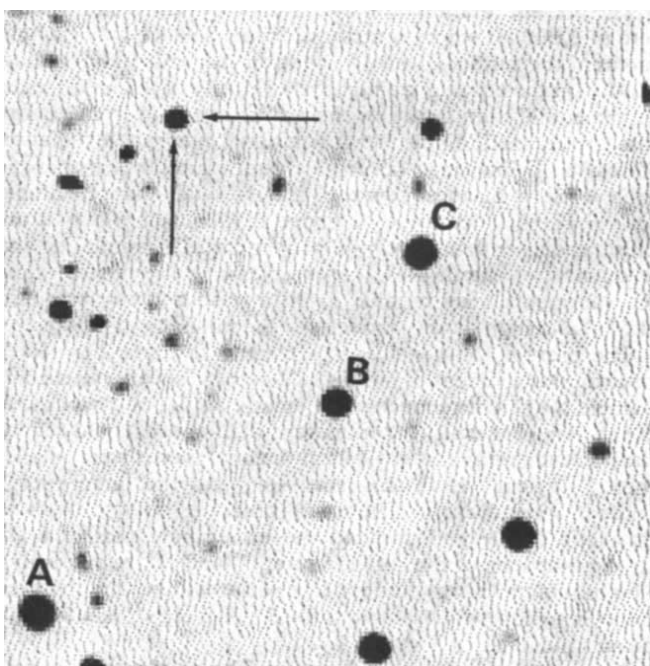


Fig. 2 Similar to Fig. 1, but strongly enhanced with a new, nonlinear technique. The field size is about 1 arc min. Faint 'objects' are not to be seriously believed.

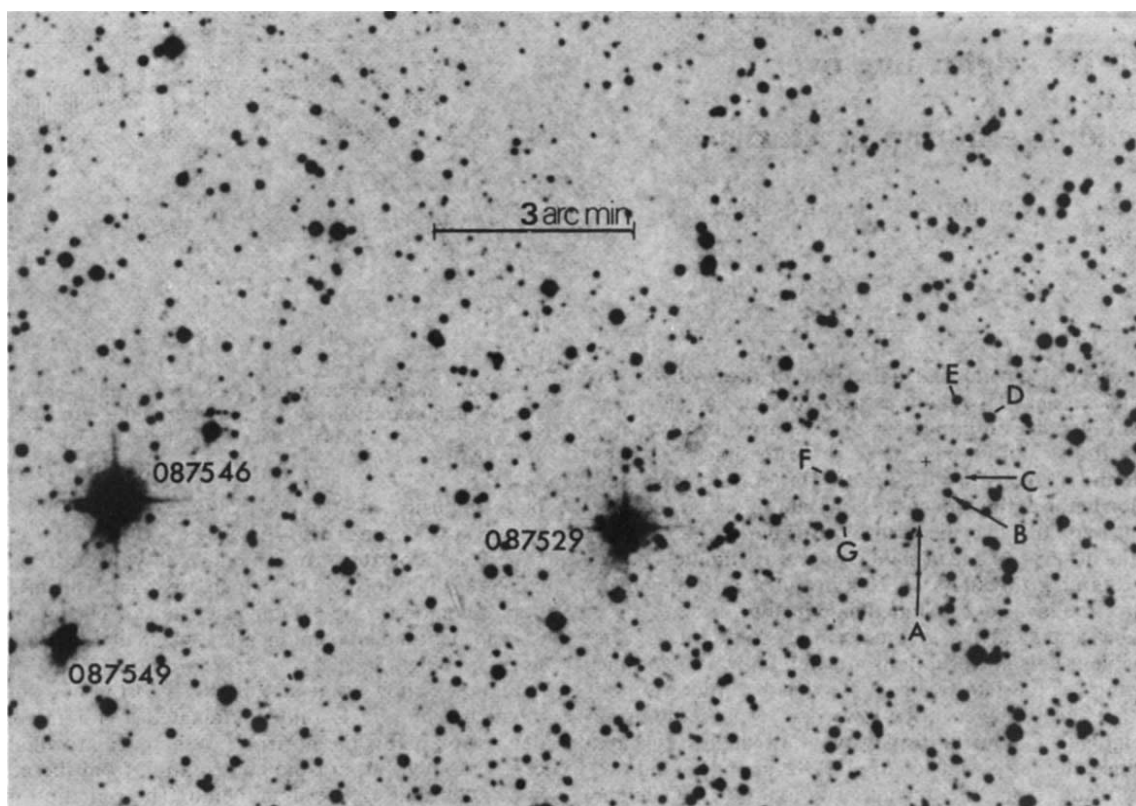


Fig. 3 Enlargement from the POSS chart E-185, showing the pulsar field, and stars denoted as in Table 1. Approximate pulsar position is marked with a cross. Horizontal bar gives the scale. North is up, east to the left. The whole Palomar chart shows high obscuration.

Table 1 lists several stars in this field, as marked in Fig. 3. Positions of the SAO stars were taken from the SAO catalogue. Stars A–G had their positions measured by J. Condon (personal communication), who quotes a random error of ~ 0.4 arc s. The radio position of the pulsar has been measured by Backer at the Very Large Array.

Pixel coordinates on the CCD stack frame were measured for stars A, B and C, as well as the pulsar candidate, and the faint object south-east from it. The relation between these (CCD) and equatorial coordinates was found, and the position of the optical pulsar candidate was derived from it. I have obtained the following differences:

$$\Delta\alpha \text{ (Condon/radio-Djorgovski)} = -1.65 \pm \sigma \text{ arc s}$$

$$\Delta\delta \text{ (Condon/radio-Djorgovski)} = +1.52 \pm \sigma \text{ arc s}$$

where $\sigma \geq 1$ arc s. This error includes Condon's quoted error of 0.4 arc s, and the estimated error of my measurements, which

is at least 0.6 arc s. There is also an unknown contribution from distortions of the CCD field (which may add as much as 0.1–0.5 arc s to this error). I believe that the realistic error bar is more like 1.5 arc s. Thus, my optical candidate is within about 1σ error box from the radio position. There is a slight possibility that the very faint object south-east from the candidate star is the correct identification; however, this object may also be spurious (a CCD artefact).

Further photometry (and, in particular, search for variability) and spectroscopy are needed. A search for optical pulses from this object has been initiated by D. Cudaback and J. Middleditch (personal communication).

Backer *et al.*¹ have determined the dispersion measure towards this source, and from it an estimate for the distance to it, ~ 2.4 kpc. It has been proposed independently by D. Van Buren (personal communication) that this source may be associated with the Vulpecula OB association, about a degree to the west. The distance to this association is estimated to be ~ 2 kpc.

This object has almost the same brightness in the present CCD red bandpass, as the faint star just north from the star C, and which is easily detectable on the Palomar red print. However, the object is invisible on the same print. The two bandpasses (CCD and POSS) are not very different, so that colour effects can hardly explain this discrepancy. It is then possible that this object had increased in apparent brightness at least a magnitude over the past couple of decades.

We thank Don Backer for ideas and stimulation, Vicki Lindsay for help in obtaining the data, and many colleagues in Berkeley for stimulating discussions. Lick CCD data taking system and flat fielding program were written by T. Laver and R. Stover. This work was partly supported through a University of California graduate fellowship.

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Table 1 Positions and offsets for the stars in the field

Star	α (1950.0)	δ (1950.0)	Star–Pulsar	
			$\Delta\alpha$ (arc s)	$\Delta\delta$ (arc s)
PSR1937+215	19 h 37 min 28.72 s	+21° 28' 01.3"	0	0
SAO-087529	19 h 37 min 48.85 s	+21° 27' 06.5"	281.0	–54.8
SAO-087546	19 h 38 min 22.48 s	+21° 27' 32.9"	750.5	–28.4
SAO-087549	19 h 38 min 25.92 s	+21° 25' 24.2"	798.7	–157.1
Condon A	19 h 37 min 29.66 s	+21° 27' 16.9"	13.1	–44.4
Condon B	19 h 37 min 27.69 s	+21° 27' 36.8"	–14.4	–24.5
Condon C	19 h 37 min 27.12 s	+21° 27' 50.8"	–22.3	–10.5
Condon D	19 h 37 min 24.90 s	+21° 28' 45.1"	–53.3	43.8
Condon E	19 h 37 min 27.01 s	+21° 29' 01.5"	–23.9	60.2
Condon F	19 h 37 min 35.30 s	+21° 27' 51.3"	91.9	–10.0
Condon G	19 h 37 min 34.60 s	+21° 27' 14.4"	82.1	–46.9

Twilight IR brightening over India due to El Chichón's eruption in Mexico

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We have observed an unusual behaviour in the twilight sky brightness in the near IR region associated with volcanic dust from the El Chichón eruption in Mexico. Approximately 8 min after sunset the sky brightness increases with time for about 8–10 min in the I band ($\lambda_{\text{eff}} = 0.87 \mu\text{m}$). This may be explained by a variation in the extinction of the illuminating solar radiation during its passage through the aerosol layer before being scattered into the observer's line of sight. Here, we estimate a mean height of 22 km, a thickness of 10 km and a drift speed of 19 km h^{-1} for the layer over Ahmedabad in June 1982.

Spectacular visual effects in the form of coloured sunsets following violent volcanic eruptions are well known^{1,2}, and the time of fading for the late twilight glow has been interpreted in terms of an aerosol layer in the lower stratosphere by using shadow heights^{3,4}. Colourful displays in the twilight sky have occurred since the latter half of May 1982 in India, and are thought to result from the recent eruption of El Chichón Volcano⁵ in Mexico.

Observations were carried out at Thaltej ($23^{\circ}03' \text{ N}$, $72^{\circ}30' \text{ E}$) near Ahmedabad using a solid state photometer in the I band ($\lambda_{\text{eff}} = 0.87 \mu\text{m}$; $\Delta\lambda_{\text{eff}} = 0.25 \mu\text{m}$) coupled to a Celestron-14 telescope. At this wavelength (located between two water vapour absorption bands), the troposphere is expected to be more transparent than the volcanic dust-laden lower stratosphere. This wavelength band is therefore ideal for detecting stratospheric dust. Figure 1 shows the time variations of the I

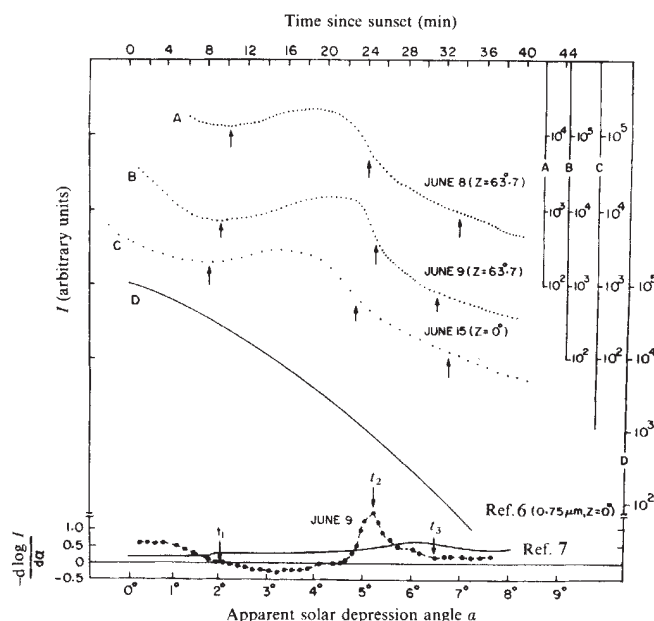


Fig. 1 Light curves A, B, C show the I band intensity (I) variation with time after sunset. D refers to the normal twilight curve at $0.75 \mu\text{m}$ (ref. 6). The plot at the bottom shows $-d/\alpha \log I$ as a function of time after sunset for the light curve B corresponding to 9 June 1982. Also shown is the derivative curve for normal twilight at $0.659 \mu\text{m}$ (ref. 7). Times t_1 , t_2 and t_3 described in the text have been indicated by means of arrows.

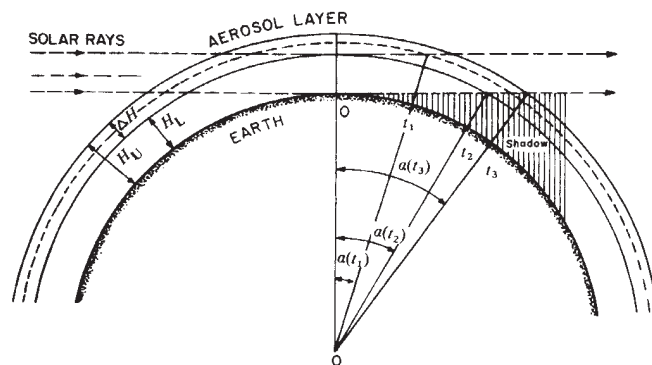


Fig. 2 Geometry of the situation for zenith observations. Solar rays illuminating the aerosol scatterers suffer attenuation during their passage through the layer.

band intensity on 8, 9 and 15 June 1982. A normal twilight curve⁶ at $0.75 \mu\text{m}$ is also shown.

A clear departure from the normal twilight drop in intensity can be seen in the form of an increase in intensity after a shallow depression occurring ~8 min after sunset. The increase culminates in a broad maximum after which it falls sharply to the normal twilight level.

Figure 2 shows that at sunset ($t=0$) the aerosol layer at a mean height ($\bar{H}$) is fully illuminated. With increasing solar depression below the horizon (α) the normal twilight intensity drops rapidly, but not the contribution from the aerosol layer which continues to be illuminated. The radiation illuminating the aerosol scatterers suffers increasing extinction with time due to its increasingly long passage through the layer. At time t_1 the central light ray has the longest passage through the layer and the extinction is at a maximum, thereby giving rise to a minimum in the observed intensity.

Due to the curvature of the Earth and the finite thickness of the layer, the total light path through the layer and the extinction are reduced after time t_1 . The observed intensity consequently increases, reaches a plateau and then falls gradually due to the increasing attenuation of light reaching the aerosol scatterers by the lower atmosphere. At time t_2 the shadow of the Earth begins cutting off the illumination to the layer from the lower end. At time t_3 , the shadow has completely cut off the layer and the intensity drops to the normal twilight level.

A model considering extinction of solar rays in the aerosol layer based on the above geometry has been evolved to explain the observed features. A uniform layer with a thickness of ΔH is considered. The extinction (A_1) suffered by the light reaching the aerosol is calculated using the relation

$$A_1(x, t) = \begin{cases} k_1 R \left\{ \left[\sin^2 \alpha(t) + \frac{2\Delta H}{R} \left(1 - \frac{x}{\Delta H} \right) \right]^{1/2} - 2 \left[\sin^2 \alpha(t) - 2 \frac{\Delta H}{R} \left(\frac{x}{\Delta H} \right) \right]^{1/2} + \sin \alpha(t) \right\} & \text{for } \alpha(t) > \sin^{-1} \left(\frac{2x}{R} \right)^{1/2} \\ k_1 R \left\{ \left[\sin^2 \alpha(t) + 2 \frac{\Delta H}{R} \left(1 - \frac{x}{\Delta H} \right) \right]^{1/2} + \sin \alpha(t) \right\} & \text{for } \alpha(t) \leq \sin^{-1} \left(\frac{2x}{R} \right)^{1/2} \end{cases}$$

where $A_1(x, t)$ is the extinction suffered by the I band intensity reaching the observer at time t after scattering by an aerosol particle located at a distance x above the lower height (H_L) of the layer. k_1 is the extinction per unit length in the I band, $\alpha(t)$, the solar depression angle at time t , ΔH , the layer thickness and R , the radius of the Earth.

The model I band intensity at time t is then given by

$$I(t) \propto \int_0^{\Delta H} \exp(-A_1(x, t)) dx$$

This line of sight integration has been numerically evaluated for various values of k_1 and ΔH . Figure 3 shows the result for $\Delta H = 10$ km and $k_1 = 3 \times 10^{-8}$ mag cm $^{-1}$. The calculated intensity passes through a clear minimum (corresponding to t_1) and then increases to reach a plateau. Despite uncertainties introduced by the unknown normal twilight intensity on which the dust contribution is superimposed, the agreement between the model and the observations in explaining the unusual brightening appears satisfactory.

The times t_1 , t_2 and t_3 used in the calculation of layer height and thickness are listed in Table 1. The times have been obtained from $-d \log I/d\alpha$ against t plots. The lower end of Fig. 1 shows a representative plot of this derivative curve for 9 June. A derivative curve for the normal twilight obtained at $0.659 \mu\text{m}$ is also shown for comparison.

Table 1 Calculation of height and thickness of aerosol layer

Date	t_1	t_2	t_3	ΔH (km)	H_L (km)	H_U (km)
	min s	min s	min s			
8 June 1982	10 20	24 12	33 22	9.4	16.5	31.8
9 June 1982	9 24	25 0	31 12	8.0	17.6	27.5
15 June 1982	8 12	23 0	32 24	9.2	17.0	36.1

The lower height of the aerosol layer (H_L) has been evaluated from time t_2 using the expression⁸:

$$H_L = R \{1 - \cos \alpha(t_2)\} \frac{\cos Z}{\cos \psi}$$

where $\alpha(t_2)$ is the solar depression angle at t_2 corrected for refraction due to a double passage through the atmosphere, Z is the zenith angle of the observation and $\psi = Z - \alpha(t_2)$. An upper limit to the height (H_U) is determined in a similar way using t_3 . The time t_1 is used in evaluating layer thickness (ΔH) from the relation (valid for zenith)

$$1 - \Delta H/2R \approx \cos \alpha(t_1) \text{ or}$$

$$\Delta H \approx R\alpha^2(t_1)$$

For zenith angles departing from zero appropriate corrections to t_1 and hence $\alpha(t_1)$ have to be made before evaluating ΔH .

The analysis yields a mean lower height of the aerosol layer $\bar{H}_L = 17.0 \pm 0.6$ km, an upper limit to the height of the layer $\bar{H}_U = 32 \pm 4$ km, and layer thickness $\Delta \bar{H} \approx 9$ km. Note that the normal summer tropopause height at 23° latitude is ~ 17 km. The observations show the unusual brightening in I ($0.87 \mu\text{m}$) and R ($0.66 \mu\text{m}$) but only an inflection in the V ($0.54 \mu\text{m}$) light curve. A spectral index $\beta(I_{\text{scat}} \propto 1/\lambda^\beta)$ evaluated from the

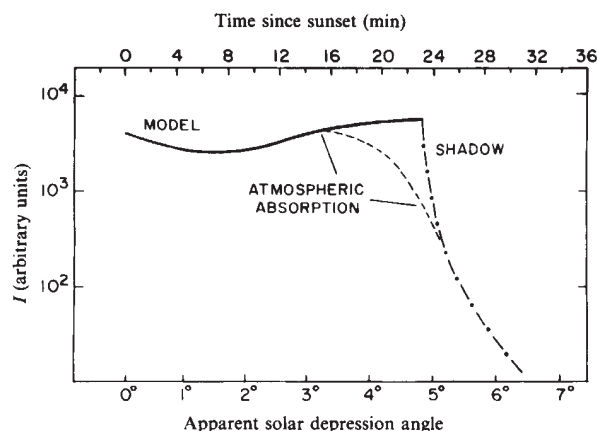


Fig. 3 A light curve computed from the aerosol layer model ($\bar{H} = 22$ km, $\Delta H = 10$ km, $k_1 = 3 \times 10^{-8}$ mag cm $^{-1}$). The effects of the atmospheric absorption (----) and the Earth's shadow (— · —) are shown.

observations and corrected for reddening due to the lower atmosphere yields a value of 3.7 at 15 min before sunset, 1.3 at the time of the peak I band intensity and 3.4 after the passage of shadow through the layer. The low value of β at the time of the peak I band intensity indicates that the radiation received at that time comes predominantly from scattering by the aerosol layer.

A lower limit to the mass of the aerosol layer M_d can be estimated by using extinction A_1 calculated by comparing the peak intensity to the intensity at time t_1 . A lower limit to the value of extinction at the time of the maximum length light passage through the layer (~ 500 km for $\bar{H} \sim 22$ km) is given by $A_1 > 0.763$ mag. This yields

$$M_d > 3.68 \times 10^{12} (f/Q) \left(\frac{a}{0.15 \mu\text{m}} \right) \left(\frac{\rho_g}{1 \text{ g cm}^{-3}} \right) A_1 \text{ g}$$

where a is the aerosol particle size in micrometres, ρ_g the grain density in g cm $^{-3}$, Q the dimensionless extinction efficiency factor and f the fractional area of the Earth covered by the layer. For a latitude spread of the layer of $\pm 20^\circ$ $f \sim 0.3$. Taking $a \sim 0.15 \mu\text{m}$, $\rho_g \sim 2$, $Q \sim 0.5$ we get $M_d \geq 4 \times 10^6$ tonnes. The earliest positive sighting of volcanic layer features occurred in Ahmedabad about 40 days after the El Chichón eruption, implying drift speed of ~ 19 km h $^{-1}$ from this delay.

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Anti-phase domain boundary tubes in Ni₃Al

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The mechanical behaviour of ordered alloys has caused much scientific and engineering interest^{1,2}. The engineering interest is due to the fact that many ordered compounds, which have unusually good mechanical properties, are incorporated as dispersed particles in and contribute to the high strengths of some high performance commercial alloys (for example, Ni-base superalloys and certain Al alloys). The scientific emphasis has been directed towards understanding the physical basis of the high strengths of these materials and anomalous behaviour such as the increase in yield strength with temperature. We report here preliminary results based on transmission electron microscopy (TEM) studies of the defect structures in the compound Ni₃Al which is the principal strengthening constituent of nickel-base superalloys used in gas-turbine engines: our observations confirm the existence of a new type of defect, anti-phase domain boundary tubes (APB) which we suspect may be an important contributor to the high strengths of these compounds.

In many nickel-based alloys the high-temperature strength is provided by the γ' precipitate based on Ni₃Al, an ordered phase with the L1₂ structure. The flow stress of the γ' phase reaches a peak value at about 700 °C. Several theories have been put forward to explain this anomalous behaviour³ with

associated TEM studies^{4,5} to characterize the dislocation configurations, but these have not succeeded in explaining all features of this behaviour. Recently, the existence of APB tubes in an Fe–Al alloy with B2 ordered structure has been demonstrated^{6,7}. The existence of these tubes had been previously predicted⁸ and they have been invoked to explain the high work hardening rates of ordered alloys by providing additional drag forces on jogged dislocations^{9,10}. In principle, APB tubes should occur during the deformation of a wide variety of ordered alloys. We therefore expect that the investigation of APB tubes in the γ' phase might provide a better understanding of the unusual deformation behaviour of this phase and give some insight into the strengthening mechanisms of Ni-base superalloys.

The early stages of this work have been carried out on polycrystalline specimens of Ni₃Al with columnar grains along the ingot axes. Ingots were prepared by melting the constituent elements and casting into 12.5 mm diameter copper moulds in a vacuum of $\sim 3 \times 10^{-5}$ torr. The ingots were remelted and directionally solidified in argon atmosphere at 10 mm h⁻¹ in a temperature gradient of 15 K mm⁻¹ followed by homogenization at 1,200 °C for 48 h.

The ingots were spark cut into rods with diameters ~ 3 mm and lengths ~ 6 mm. The rods had the columnar grains oriented along their axes. These specimens were compressed 3–5% at one of the following temperatures: room temperature, 200 and 400 °C. Thin foils for TEM work were prepared from slices of the deformed rods; the final electropolish was carried out in a solution of 5% perchloric acid in ethyl alcohol at –50 °C and 15 V. The foils were examined in a JEOL JEM 100B microscope fitted with a top entry stage which allows $\pm 30^\circ$ of tilt. The 'weak beam' technique with superlattice and fundamental reflections was used.

The following results were obtained from a specimen with surface close to the (112) plane. The sample was compressed at 400 °C to $\delta = 4.1\%$. Figure 1 shows weak beam images of the same area using three non-coplanar fundamental

reflections. Contrast experiments have proved that the stacking faults in these pictures are $\langle 112 \rangle$ type. Figure 2A shows dotted lines parallel to [011] or [101] in this area in a semi-weak beam image when the superlattice reflection $02\bar{1}$ was used; the deviation parameter w was ~ 21 ($\sim 2g$ was satisfied). These features are not apparent in the fundamental reflection modes (Fig. 1). Figure 2B is a superlattice reflection weak beam image of the same area. The reflection used was $20\bar{1}$. The deviation parameter here is $w \sim 42$ ($\sim 3g$ was satisfied).

The lines in Fig. 2B are longer than the corresponding lines in Fig. 2A due to different tilts of the specimen. From the position of Kikuchi lines relative to the diffraction spots, it was possible to determine (1) the sense of the tilting and (2) from the observed length changes, that these lines are along (011) and not [101].

The contrast shown by the lines can be summarized as follows: (1) The defects are along $\langle 011 \rangle$, the Burgers vector direction in the material. (2) The defects are visible only when superlattice reflections are excited. (3) The oscillatory contrast in Fig. 2A and B shows that the defects are line defects. (4) The fringes are symmetrical about the centre of the specimen and no fringes occur when $w = 0$; this latter effect is characteristic of the contrast from two overlapping APBs⁶. (5) The width of these defects does not change with deviation parameter.

All of these characteristics are as expected for APB tubes. It can be established unambiguously that these defects are not dislocations as they are invisible in any of the three non-coplanar fundamental reflection pictures (Fig. 1) which should image all possible dislocations. Again, the features can not be Lomer–Cottrell (LC) locks since although in f.c.c. structures these are along $\langle 011 \rangle$ directions (those with $\mathbf{b} = a/2 [01\bar{1}]$ are along $[011]$) they should be visible in Fig. 1A and C, since in these cases the invisibility criterion $\mathbf{g} \cdot \mathbf{b} = 0$ does not hold. In fact LC locks are edge dislocations and they should also be visible in Fig. 1B, because the $\mathbf{g} \cdot \mathbf{b} \times \mathbf{u} = 0$ criterion does not hold, even though $\mathbf{g} \cdot \mathbf{b} = 0$ in that case. Neither can the defects be normal anti-phase domain boundaries. If they were two

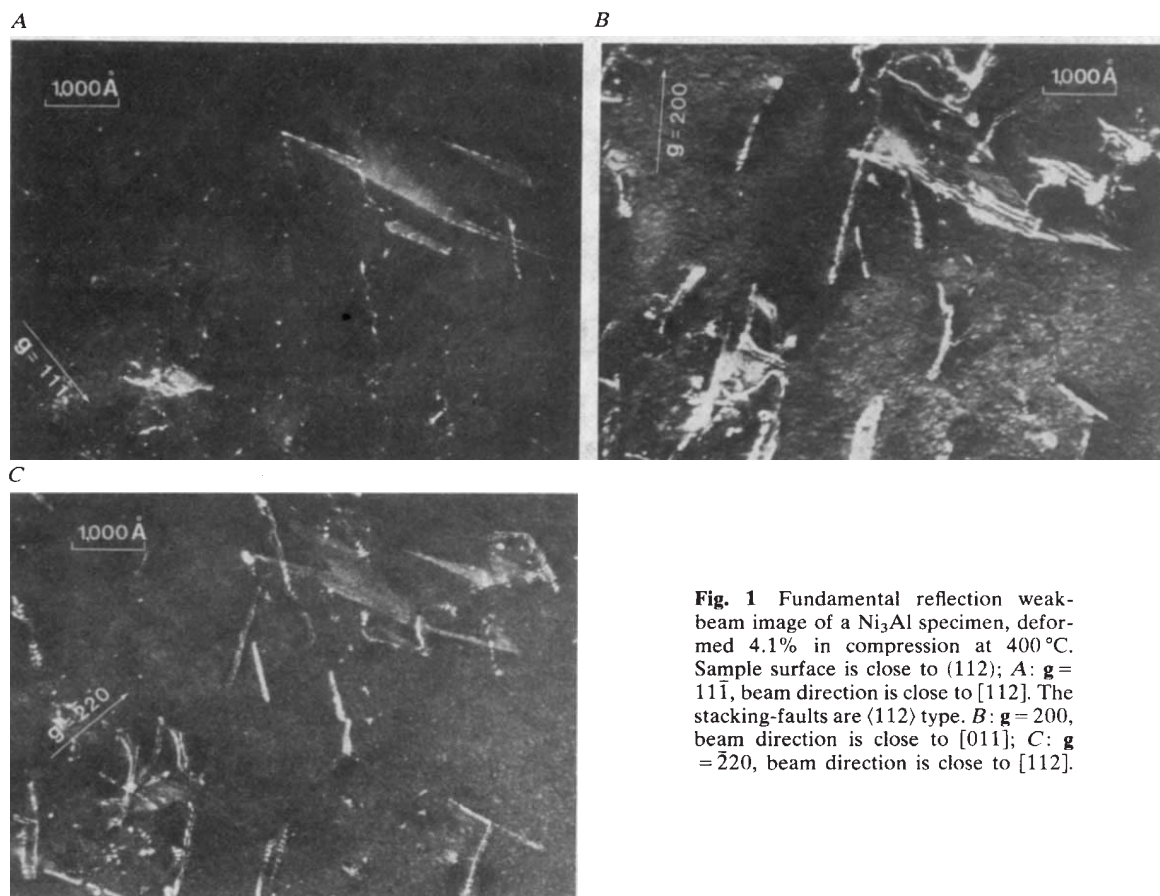


Fig. 1 Fundamental reflection weak-beam image of a Ni₃Al specimen, deformed 4.1% in compression at 400 °C. Sample surface is close to (112); A: $\mathbf{g} = 11\bar{1}$, beam direction is close to $[112]$. The stacking-faults are $\langle 112 \rangle$ type. B: $\mathbf{g} = 200$, beam direction is close to $[011]$; C: $\mathbf{g} = 2\bar{2}0$, beam direction is close to $[112]$.

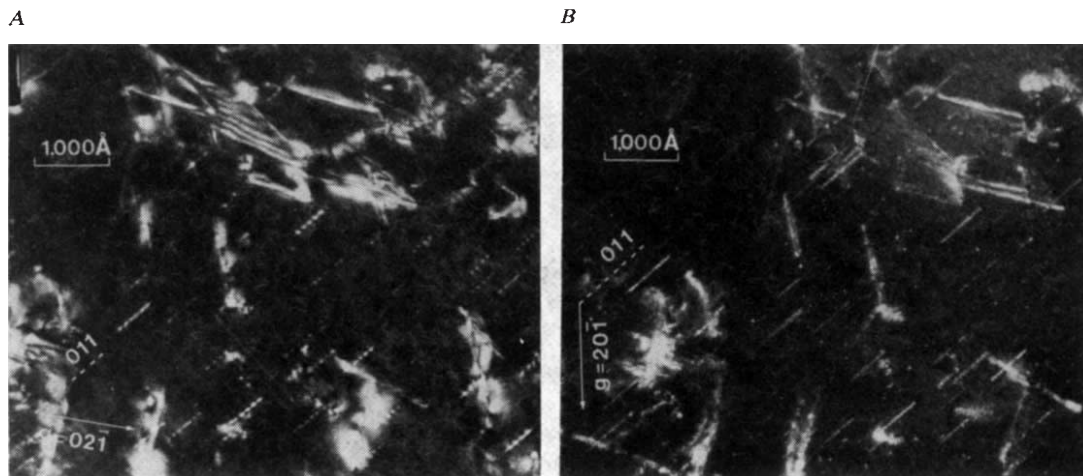


Fig. 2 Superlattice reflection weak-beam image of the same area as that in Fig. 1A: $g = 02\bar{1}$, $w \approx 21$; beam direction is close to $[548]$. B: $g = 20\bar{1}$, $w \approx 42$, beam direction is close to $[326]$.

dimensional APBs they should not show depth oscillations as in Fig. 2. If they were narrow strips of APBs, they should be connected to partial dislocations, but the latter are strongly imaged using the fundamental reflections. The above discussion leads to the conclusion that the defects along $[011]$ in Fig. 2 are APB tubes.

APB tubes have now been found in deformed ordered alloys with B2 (refs 6, 7) and $L1_2$ superlattices, suggesting that they may be a general feature in deformed ordered alloys. On the other hand their importance in affecting mechanical properties

remains to be assessed. Experiments are under way to determine *inter alia* whether the peak in the curve of flow stress as a function of temperature may be associated with the temperature at which climb helps jogs in edge dislocation to align, and in screw dislocations to move non-conservatively, and how APB tubes play a role in superalloys with large proportions of γ' phase.

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A lightning-associated phenomenon and related geomagnetic measurements

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Many observations of phenomena associated with lightning have been made at a considerable distance. Apart from the element of personal danger from direct strike, wind and rain make field conditions unfavourable for observing, and the phenomena are usually very transient. We report here a phenomenon associated with lightning which was observed indoors and at close range following a lightning strike. Magnetic field measurements in the room where the phenomenon was seen are also reported.

In the late evening of 8 September 1981 a thunderstorm approached Te Ngaere ($35^\circ 01' 24''S$, $173^\circ 52' 10''E$), 300 km north of Auckland, New Zealand. The one-storey house at Te Ngaere, where the observation was made by Mrs E. V. Sale, is one of a row built behind a sandy beach and occupies the highest point of a low sand-bar at the mouth of a swampy valley. The house is constructed of timber with a cement-tiled roof and steel-reinforced concrete floor. The living-room has a partition (behind the piano in Fig. 1) forming an entry way about 2 m wide. There is a French door with a corded woollen mat just inside. The floor is covered with felt-lined linoleum. Mrs Sale had brought in some wet steel tools, which she had roughly dried and laid out to dry fully on a doubled sheet of

newspaper 60 cm by 40 cm (Fig. 1). She was kneeling at the east side of the paper bending over the tools when another heavy lightning strike occurred, just outside the house. Mrs Sale is an amateur astronomer and impressed us as a reliable observer. She related the events as follows:

The next lightning seemed directly overhead and very bright and was accompanied by a simultaneous very loud clap of thunder. I looked up as the whole house shook and then looked down and saw a flow of light come in under the door. It settled in a blob near the edge of the area where the tools were laid out. It was not in any true shape but about 3 or 4 inches long and 2 inches wide, moving along the floor, less than half an inch thick, seemingly fluid in shape and texture. It reminded me of quicksilver, being a bluish-silver colour and it had rounded sides like a blob of mercury. It was brighter at the edges than in the middle, but it did not seem, especially in the light of the room, to glow, nor did it give out sparks.

From the central body arms flowed out like runs of oil among the tools. The trails weaved through the tools—not actually over them but round them—moving back into the main body of the blob and then going out doing the same kind of movement over again. There was no sound or smell. The arms finally all went back into the blob which disappeared again suddenly out under the door. There was no bang and when I ventured to touch the tools there was no charge on them.

We visited the house on 16–18 November 1981. From this visit together with subsequent correspondence, a more detailed account can be given.

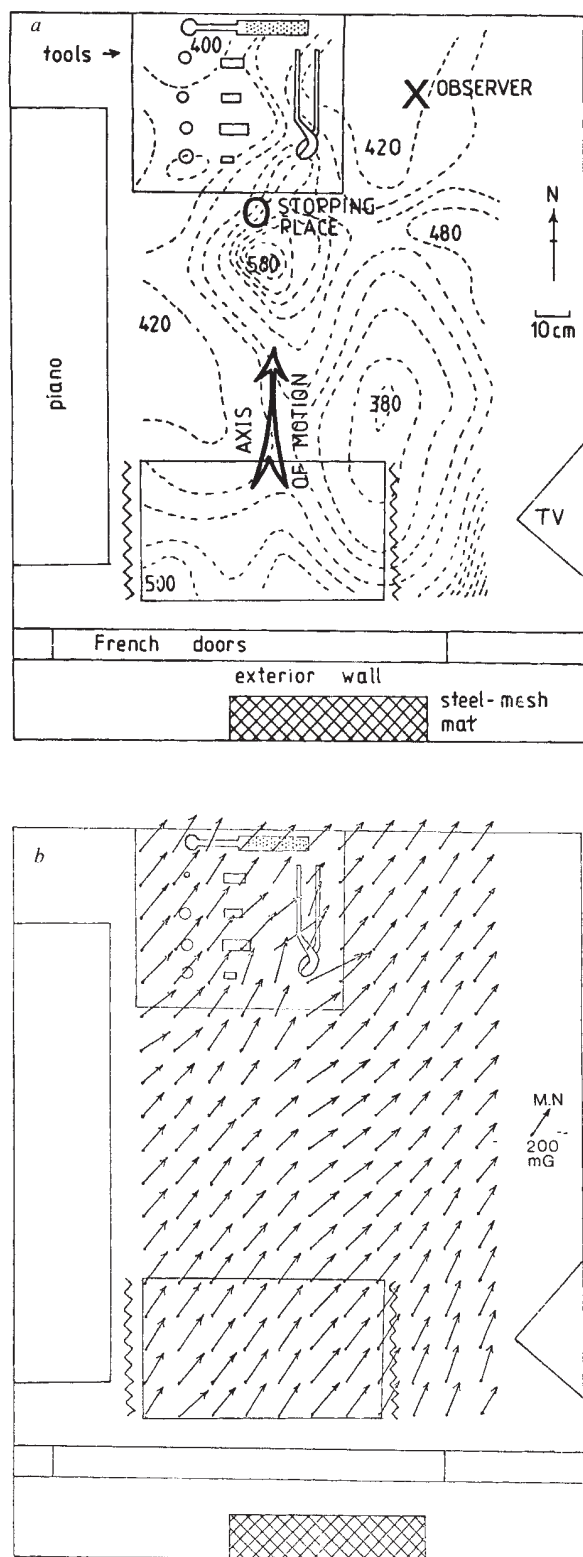


Fig. 1 *a*, Sketch of the region in which the body was observed, showing the observer's position and the path of the body. The vertical magnetic field intensity in milligauss is superimposed, with a contour interval of 20 mG. *b*, Horizontal component of magnetic field, over the same area. Intensity scale is given on the right.

The body was opaque, and must have looked like a faintly luminous blob of milky glass. No trace of it was left anywhere. A strike point could not be found for the lightning and there was no sign of damage to the exterior television antenna,

vertically above the set in Fig. 1, or to a large pohutukawa tree a few metres south-east of it. The door was found to have a tapering vertical gap near the bottom, close to the floor about 10 cm long and 0.5 cm wide.

The lighting in the area of the living-room was dim and indirect, coming mainly from a shaded 60 W standard lamp about 4 m away. In an experiment Mrs Sale felt that a blob of freshly poured lead replicated the luminosity of the object when it was illuminated by a candle 10 cm away. We estimate the power emitted as light therefore as $\sim 2 \times 10^{-4}$ W; no noticeable heat was emitted. Two timing measurements were done as Mrs Sale rehearsed the motion of the body by pointing. The two agreed, giving 4 s for each travel and 7 s halt, a total of 15 s.

As the phenomenon occurred during a thunderstorm and immediately after a heavy strike of lightning in the vicinity, it may be related to that broad group of events currently classified as 'ball lightning'¹⁻³.

Magnetic fields in the vicinity were measured with the tools in place on 17 November 1981. This may be of particular interest as although many accounts of ball lightning refer to iron objects such as axes, stoves and tramlines only one previous author⁴ has explored magnetic effects. The geomagnetic field at floor level was mapped near the path of the body with a laboratory fluxgate magnetometer (Schoenstedt DM-2220). Three-axis measurements were facilitated by setting the unidirectional probe in a wooden cube with 6.3-cm sides and taking readings with successive orthogonal faces of the cube resting on the floor and squared against a wooden rule. Each of the observed elements varies over the floor by $\sim 20\%$ which is not unusual for steel-reinforced concrete.

The ambient geomagnetic field is steeply dipping at about 60° above horizontal, and the body was evidently constrained to move in the horizontal plane. If the body contained free dipoles such as current loops, then after alignment by the field it would be expected to experience a force towards the region of most intense vertical field, and possibly move in that direction. It will be seen in Fig. 1 that there is a close correspondence between the path of the body and the 'ridge' of most intense vertical magnetic field. These observations are therefore consistent with the body containing free magnetic dipoles of some kind.

Since the path of the body is a little offset from the line of vertical-component maxima, a capricious force such as a current of air is implied, which might also explain the retirement of the body. It seems reasonable to attribute to draughts the major action in moving the body, the initial entrance and wavering shape strongly suggest this. The living-room is large and well ventilated so that significant and very variable draughts might be expected at floor level given the rapidly-changing winds which would accompany a thunderstorm. Mrs Sale plainly regarded the body as being liquid because it had a surface with wavering blob-like outline and must have been denser than air.

No firm conclusions as to the nature of the body seem possible. The close association with lightning suggests that the strike was the prime cause. The body appears to have been denser than air, had a surface like a liquid, although it was non-wetting, and had a milky-blue luminous appearance. Its radiant energy was in the order of 2×10^{-4} W, observed for 15 s. Wind draughts seem to have been the dominant transport mechanism. From mapping the geomagnetic field we found a weak correlation between the motion of the body, and the regions of most intense vertical field, as would be expected if the body contained free magnetic dipoles or current loops.

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Extensive underthrusting of undeformed sediment beneath the accretionary complex of the Lesser Antilles subduction zone

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Along the eastern margin of the Caribbean Plate, which overrides the Atlantic oceanic lithosphere at $\sim 20 \text{ km Myr}^{-1}$ in an easterly direction¹, east of the Lesser Antilles island arc, lies a wide accretionary complex (Barbados Ridge Complex); 260-km wide at the latitude of Barbados and $>20\text{-km}$ thick beneath Barbados^{3,4}, it increases in width and thickness from north to south² (Fig. 1). The style of deformation varies considerably along the eastern margin of the complex^{2,3,5-11}, and is related to the thickness and type of sediment on the Atlantic Ocean floor. A common feature is that a proportion of undeformed sediment from the ocean floor is brought beneath the deformed sediments of the accretionary complex, and in some areas a clearly defined seismic reflector separates the deformed and undeformed sediments, which has been interpreted as a decollement surface^{5,12,13}. A seismic section from an investigation of the accretionary complex, conducted from RSS *Discovery* in 1980 using 12-channel seismic reflection and long range side-scan sonar (GLORIA), shows undeformed sedimentary rocks carried horizontally 74 km beneath deformed rocks forming the accretionary wedge. We report here that the low shear stresses required on the decollement between the deformed and undeformed rocks are made possible by porewater at pressures close to that of the lithostatic load.

The longest of three seismic profiles run across the eastern margin of the complex 50 km north of DSDP Leg 78A, Site 543 (X-X', Fig. 1) shows that a reflector with layered reflectors between it and the rough surface of the oceanic basement below,

extends without significant interruption for nearly 45 km from the edge of the complex beneath a westward thickening wedge of material characterized by disturbed discontinuous reflectors (Figs 2 and 3). Beyond a region of disturbance, a reflector can be seen again continuing above the oceanic basement to the end of the section, some 74 km from the edge of the complex. This reflector and its apparent continuation are interpreted as a decollement, and the implication of this is that virtually undeformed sediment is carried on the ocean crust beneath the overlying accretionary complex for at least 45 km, and probably more than 75 km. Similar 'decollement' reflectors have been reported from other forearc accretionary complexes where sediment entering the complex on the ocean floor is of at least moderate thickness¹⁷⁻¹⁹. The previous greatest landward extent of such a reflector, 32 km from the edge of the accretionary complex²⁰, was demonstrated at the Nankai Trough off southern Japan. Extensive decollements are also well known in mountain belts on land²¹⁻²⁴.

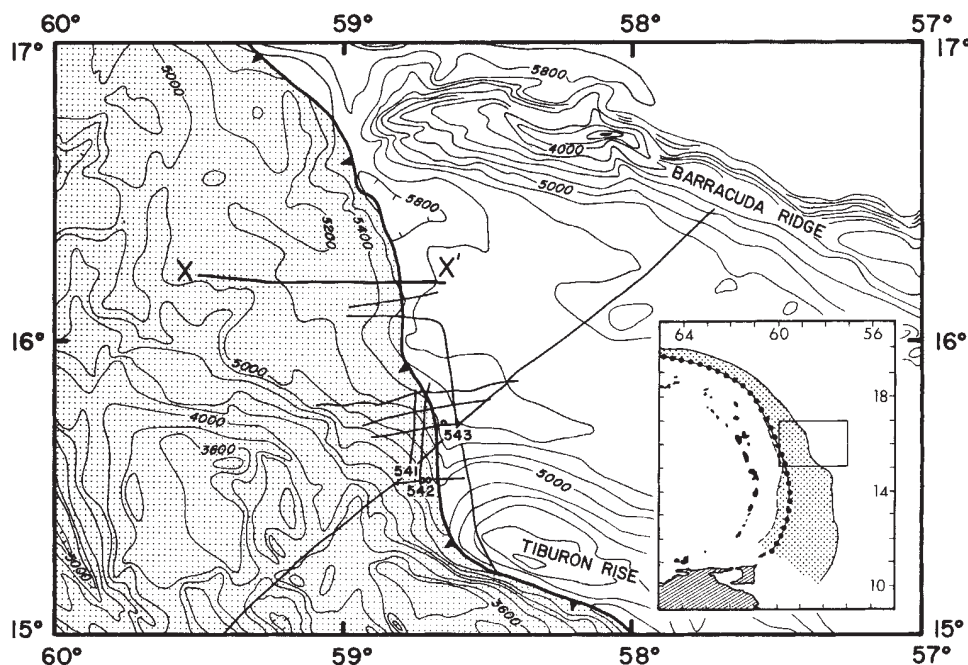
It was the principal objective of DSDP Leg 78A in 1981 to drill through the accretionary complex near its leading edge, penetrating deformed sediment, the decollement, the undeformed sediment sequence, and finally the oceanic igneous basement¹⁴⁻¹⁵. Three sites were drilled on the northern flank of an oceanic basement ridge (the Tiburon Rise) where relatively shallow bathymetry and thin sediments provided favourable conditions (Fig. 1). The decollement was successfully reached in Hole 541, but poor drilling conditions prevented penetration of the undeformed sequence underneath.

The presence of a clearly recognizable decollement constrains interpretations of the formation of the accretionary wedge above it, because the thickness of sediment entering is known, and no further sediment is added from beneath, where the decollement is unbroken. This is important for calculations relating volume of the accretionary wedge and uplift of the surface to the rate of sediment input²⁵. Currently, only the upper third of the sedimentary sequence on the Atlantic Ocean floor is added to the leading part of the complex, and if the decollement is stratigraphically controlled, a smaller proportion was added in the past.

East of the accretionary wedge, no clear difference can be seen in the character of the seismic section above and below the reflector which becomes the decollement, but drilling at Site 543 showed that it seems to lie at a change from Lower Eocene mud to radiolarian claystone, and also corresponds to an increase in the cohesion of the rocks from 40 to 100 kPa.

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Fig. 1 Map of the front of the accretionary complex of the Lesser Antilles subduction zone, east of Guadeloupe. The area of the complex is stippled, and its front is shown by a heavy line. Also shown are multichannel seismic reflection lines including that shown in Figs 2, 3a, b (X-X'), and the drill sites from DSDP Leg 78A. Bathymetric contours are at 200-m intervals. The inset shows the area of Fig. 1 in relation to the whole of the accretionary complex (stippled) and the Lesser Antilles island arc (black island shapes). The axis of negative gravity anomaly is shown by dotted line.



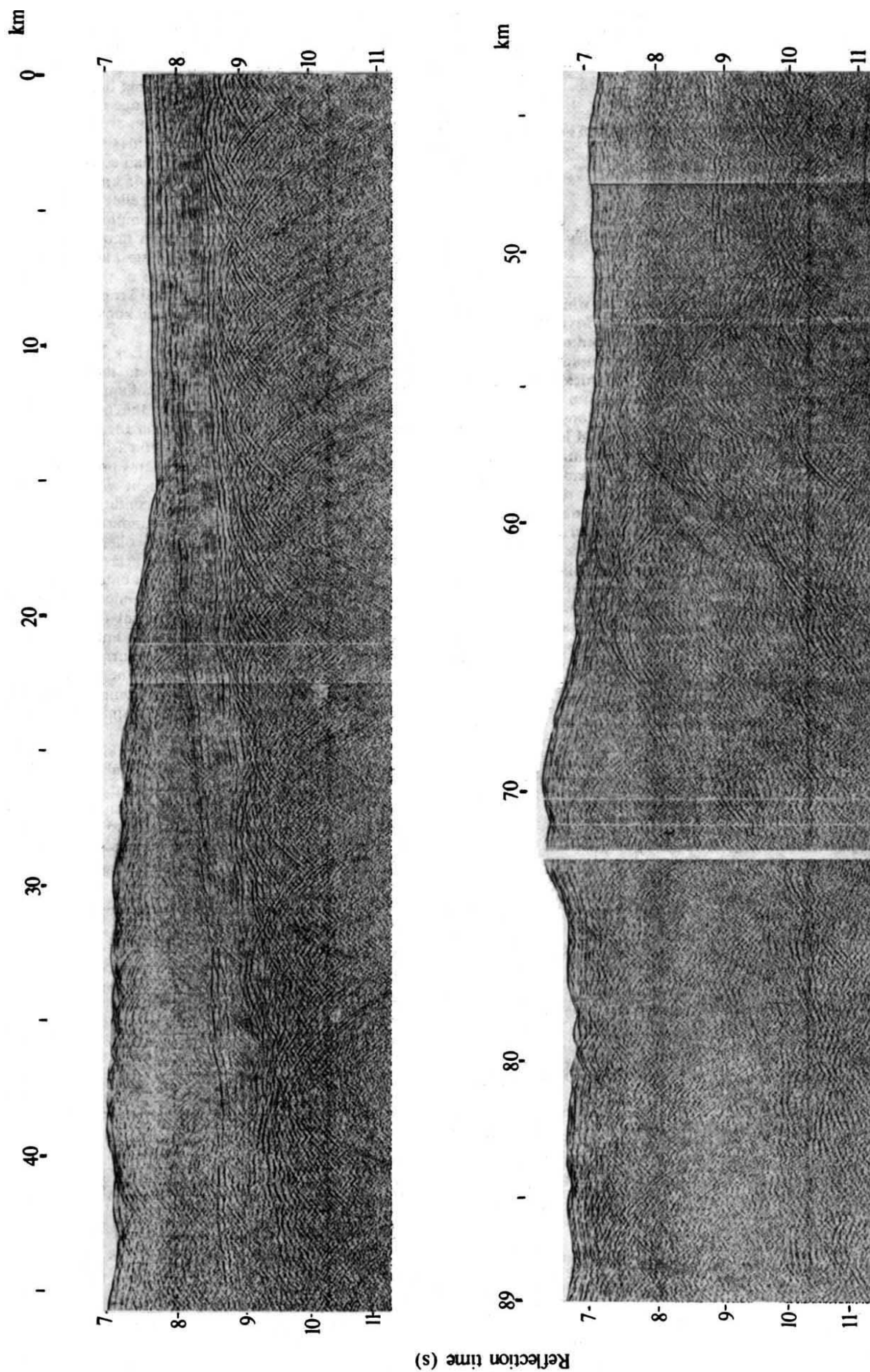


Fig. 2 A 12-fold stacked and deconvolved seismic reflection section across the front of the accretionary complex (X-X' in Fig. 1). The oceanic basement can be seen beneath the complex, easily recognized from its rough surface which propagates prominent hyperbolae. Above this, another prominent reflector, interpreted as the decollement surface above a sequence of undeformed sediments, can be traced to nearly 60 km from the right-hand end of the section where it becomes obscure, but between 75 and 89 km it can be seen again above the basement, giving a total length of 74 km from the edge of the complex.

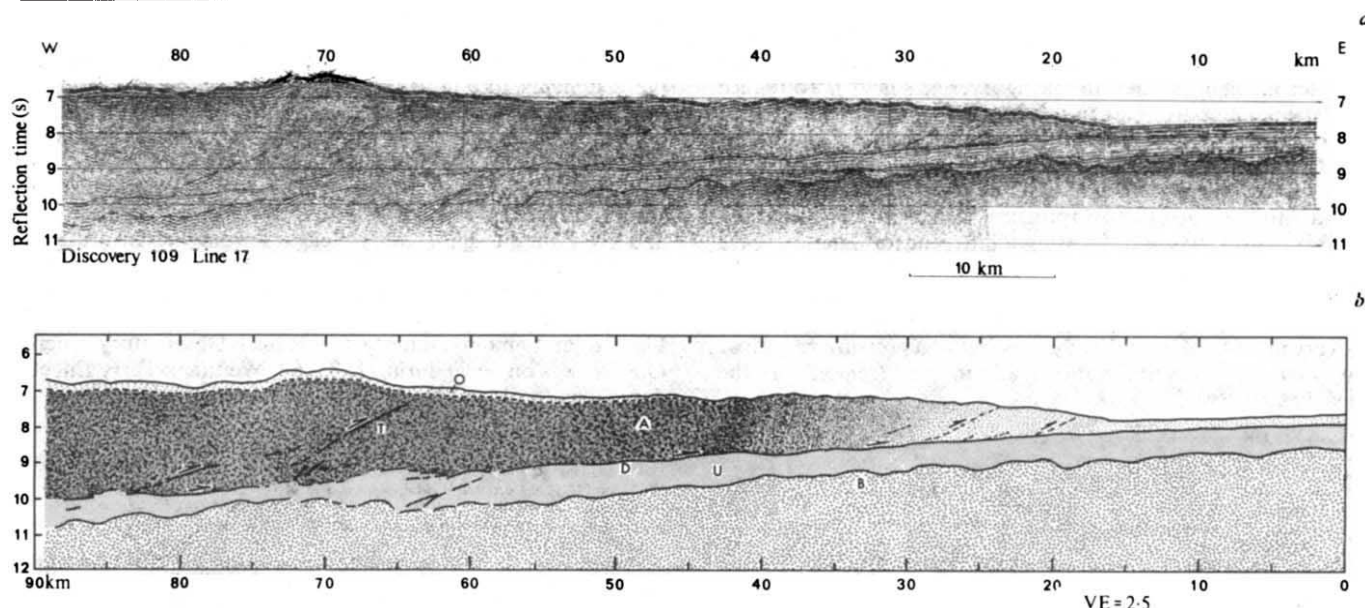


Fig. 3 *a*, Migrated version of Fig. 2. Most, but not all, of the more prominent reflectors have been highlighted. Of particular importance, apart from the basement and decollement, are the two prominent westward dipping reflectors, between 60 and 85 km from the right. They and other shorter dipping reflectors between 15 and 20 km from the right are interpreted as thrusts. *b*, An interpretational cross-section of the accretionary complex, based on Figs 2 and 3*a*. The wedge of accreted and deformed rocks (A) thickens rapidly over the first 25 km, where rapid compaction and movement along closely spaced thrusts, which are more abundant and closely spaced than the few depicted, appear to be the dominant deformational processes. The wedge retains a nearly constant thickness for another 20 km until it increases thickness again by movement along the widely spaced major thrusts (T), which produce uplift of the seabed. Beneath the decollement (D) sediment is undisturbed (U) until it reaches the region of the major thrusts, where some of it may be stripped off into the wedge. The dipping reflectors shown just above the basement (B) between 60 and 65 km are probably also reflections from a basement high slightly out of the plane of the section. Above the complex lies a thin layer of sediment (O) which was deposited directly on to it. The increase in the density of the ornament of the accretionary wedge reflects the inferred increase in density of the rocks, resulting from tectonic compaction. Vertical exaggeration of the relief of the seabed is 3.3:1. The average vertical exaggeration of features in the rocks beneath the seabed is 2.5:1.

The western end of the section is ~68 km east of the axis of the trough in the basement beneath the accretionary complex, revealed by gravity and seismic refraction data, where it is inferred that the ocean crust passes beneath the crystalline crust of the Caribbean Plate, and so it is not possible to say whether the layer of undeformed sediment is taken deeply into the subduction zone proper or stripped off and added to the base of the accretionary complex.

The rate of thickening of the accretionary wedge is greatest at the leading edge, where the angle of its taper is 5.4° , and decreases arcwards until, between 38 and 54 km from the right of the section there is very little significant increase in thickness with a taper angle of 1° (Fig. 3*b*). There are probably two main mechanisms for the initial thickening: imbricate stacking of small thrust slices, and direct horizontal shortening of the sediments accompanied by the expulsion of porewater during tectonic compaction. Thrusts with a vertical spacing of ~150 m were encountered at Sites 541 and 542 of DSDP Leg 78A, and their presence in the section is indicated by poorly defined westward dipping reflectors near the front of the wedge (Figs 2 and 3*a*). The small scale of the structures at the toe of the complex makes them difficult to recognize on the seismic section which, with a trace spacing of 50 m, does not resolve well reflectors shorter than 0.5 km. The initial thickening of the wedge will tend to reduce the deviatoric horizontal stress acting in the wedge, and it is probably accompanied by an increase in the strength of the rocks as they compact, so that ~22 km from the front some balance seems to be achieved with the rocks forming the wedge able to withstand the stress without any large increase in strain. Further significant thickening of the accretionary wedge seems to proceed by movement along much more widely spaced thrusts such as that seen some 50 km from the front of the wedge, but probably with little change in the strain of the rocks within the thrust slices (Fig. 3*b*). The point at which the wedge begins to increase in thickness again is also close to where the decollement first appears to suffer disruption.

There is little direct evidence on the state of compaction of the rocks forming the wedge. The rocks sampled at DSDP Sites 541 and 542 (refs 14, 15) showed a small but significant increase in density of ~13% from their equivalents at the reference Site 543 in the undeformed sequence in front of the complex, with an accompanying decrease in porosity, but no significant change in seismic velocity. An analysis of the stacking velocities used to produce Fig. 2 and the most effective velocities required to produce the migrated section of Fig. 3*a* was unable to determine any significant change in the seismic interval velocity of the rocks forming the wedge with distance from the front, other than what might be expected from the increase in thickness. This approach was of limited accuracy, however, because of the relatively small aperture of the seismic streamer compared with the depth of the reflectors, and so only a very strong lateral change in velocity is ruled out. A study of sonobuoy data in this area²³ also failed to show any strong lateral variation in velocity.

The small angle of taper between the top and bottom surfaces of the accretionary wedge and the low strength of the rocks forming the wedge and the underlying sequence^{14,15}, indicates that the shear stresses on the decollement must be very low²⁷⁻²⁹. The dynamics of a thrust wedge in which the stresses in the wedge exceed its strength have been analysed by Chapple²⁷, who derived the following relationship, where the angles of slope of the base and surface are small:

$$\begin{aligned} \text{Shear stress at base of wedge} &= \text{Density} \times \text{Gravity} \times \text{Angle of slope of surface} \\ &\quad \times \text{Thickness beneath a horizontal surface through the tip} \\ &\quad \text{of the wedge} \\ &\quad + 2 \times \text{Yield stress of wedge} \times \text{Angle of slope of base.} \end{aligned}$$

Applying this formula to the region of the wedge with a taper of 3° between 21 and 38 km, assuming that rocks are saturated with water but not overpressured, and behave as a Moho-Coulomb material with an angle of friction of 30° , the shear stress on the decollement is computed to be 0.44 MPa. This is

much less than the 8.5 MPa expected from the load imposed by water saturated sediments and a coefficient of friction on the decollement of 0.85 (following Byerlee's law³⁰). To reduce the stress, the porewater in the zone of the decollement must be overpressured³¹ to a value which is 0.97 of the load which would be imposed by the wedge if no water were present. If clay minerals reduce the coefficient of friction on the decollement³² to 0.2, then the overpressure would be 0.81 of the dry wedge load. Davis *et al.*²⁹, using a different formulation, have obtained very similar values for overpressured porewater in the Barbados Ridge Complex and other accretionary wedges. These estimates of porewater pressure are corroborated by the discovery in DSDP Hole 542 of porewater at a pressure very close to that of the lithostatic load in the region of the decollement^{14,15}.

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Rainfall over the Arabian Sea during the onset of the 1979 monsoon

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One path to understanding the summer monsoon of south-east Asia lies through its regimes of rain. There the main scales of interest range, on the small side, down at least to component rainstorms: that is, extending over hundreds of kilometres and lasting about a day. On the large side, interest might be extended to include the whole quasi-planetary monsoon system, although here, for practical reasons, the limits are taken to be 1 week and several thousands of kilometres. The main branch of the summer monsoon forms over the Arabian Sea. The rainfall of this sea, roughly 3,000 km in its dimensions, is known to us from about 40 coastal stations, mostly in India, plus six instrumented islands and the reports of seagoing ships. The ships stay mainly in a few lanes and in any case only record the frequency of rain, not the amount. Thus, even taken all together, these reports are hardly adequate for building a picture of the monsoon rains. We report here a new technique for inferring daily rainfall from visible or IR images obtained by a geostationary satellite, applied in a simplified form to the Arabian Sea during the onset of the 1979 summer monsoon. The rain area estimated increased spectacularly (from 1 to 14%) over a 1-week period coincident with the appearance of the Somali jet and development of the onset vortex.

Some scientists have exploited low-orbiting satellite observations of the Arabian Sea: C. S. Ramage, using visible imagery (personal communication), and Kidder and Vonder Haar and Rao *et al.*, using microwave data^{1,2}. In each, the scale of the estimates—5 days or more—is too long to be of much use in portraying the monsoon through its development.

We conclude that this long decollement only functions as consequence of the overpressuring of porewater contained in the sediments forming the accretionary complex and undeformed sequence beneath it.

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The 1979 Monsoon Experiment, which had an Arabian Sea component, may afford the best opportunity yet to build an evolutionary picture of the onset rains. The basis for this claim is the Indian Ocean Geostationary Operational Environmental Satellite (GOES IO). This satellite, positioned over the Equator at 60°E, returned a visible and/or IR image, of 1–8 km resolution, once in almost every hour of the days from 11 to 21 June, a period which coincides almost perfectly with the onset of the 1979 monsoon.

To map daily rainfall over the Arabian Sea as the 1979 summer monsoon became established, drawing on several studies (see refs 3 and 4), we hypothesize the following: (1) four rainrates (nil, light, moderate and heavy) can be inferred consistently at any hour from a sequence of hourly visible or IR images; (2) the nil-rate class predominates; (3) most nil and heavy cases can be decided solely on the basis of brightness; (4) time change of brightness and pattern are important in the remaining cases; (5) most of the data in a single image are redundant; and (6) visible and IR data are complementary.

One special constraint follows from the enormous volume of data generated by the GOES IO imaging instrument; we cannot look at every element of every picture. In fact, this kind of problem could only be addressed by training a machine to take repeated measurements and make simple decisions.

The approach that has been taken is to imagine a network of gauges, distributed over the basin of the Arabian Sea. For each hour at each gauge a meteorologist, assisted by a computer, infers from satellite pictures whether the rainfall was light, moderate, heavy or nonexistent. The sum over 24 h of these rainrate class assignments yields an estimate $\hat{R}$ of the daily rainfall R . Thus, if $\hat{r}_i$, $i = 0, 1, 2, 3$, is the estimate of hourly rainfall attributed to the i th rainrate class and f_i is the frequency of the i th rainrate class over 24 h,

$$R \approx \hat{R} = f_0\hat{r}_0 + f_1\hat{r}_1 + f_2\hat{r}_2 + f_3\hat{r}_3$$

or, since $\hat{r}_0 \approx 0$

$$\hat{R} = f_1\hat{r}_1 + f_2\hat{r}_2 + f_3\hat{r}_3$$

Each $\hat{r}$ is a constant to be determined by multivariate least-

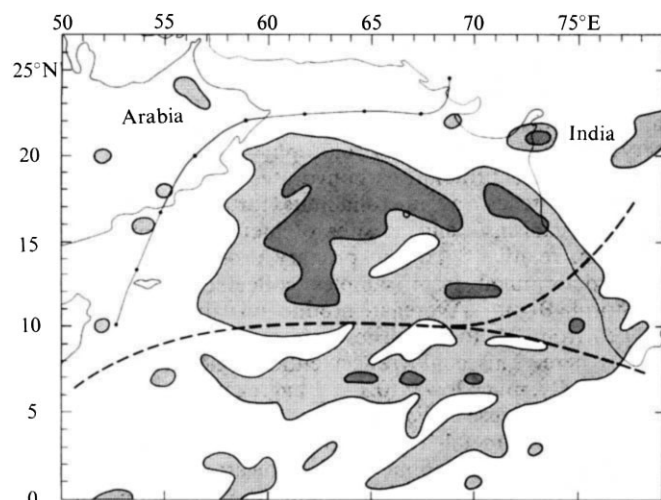


Fig. 1 Rain frequency for 17 June 1979. Stippling marks the grid points where rain was registered at least once during the day. The darker stippling shows where the heavy rain class was recorded at least once. Also shown are the axis of the Somali jet (dashed line) and the centre of the onset vortex (circle, at 16° N, 67° E), both from ref. 7 and the edge of the monsoon inversion (knotted line), from ref. 9.

squares regression of $\hat{r}$ on R , where R is measured by a gauge or radar.

Rain is estimated from a sequence of Earth-located (navigated), registered satellite pictures, each image of the sequence being spaced an hour or so from the others. There may be an IR sequence and a visible sequence or either alone. Images are displayed and analysed on the University of Wisconsin Man-computer Interactive Data Access System, McIDAS⁵.

Rain has been estimated by this so-called grid history technique for the area from the Equator to 28° N and from 50° E to 79° E. Initially, the resolution of the grid across this area was 0.5°. It has since been relaxed to 1°. Eight days have been processed, 11–18 June 1979.

Applications of this technique to parts of the South China Sea and the eastern Atlantic Ocean when gauges or radars were present suggest values for the coefficients in the ratio of 1:4:10. So far it has not been possible to acquire enough ground observations of rain around the Arabian Sea to run the regression there. Thus, only rain class frequencies are presented here.

A photographic view of rain frequency for 17 June 1979 is shown in Fig. 1. At this stage of the monsoon, rain was extensive over the Arabian Sea but largely absent over India. A band of mostly light to moderate rain, centred near 7° N, angled north and north-west along the Malabar coast of India, then merged

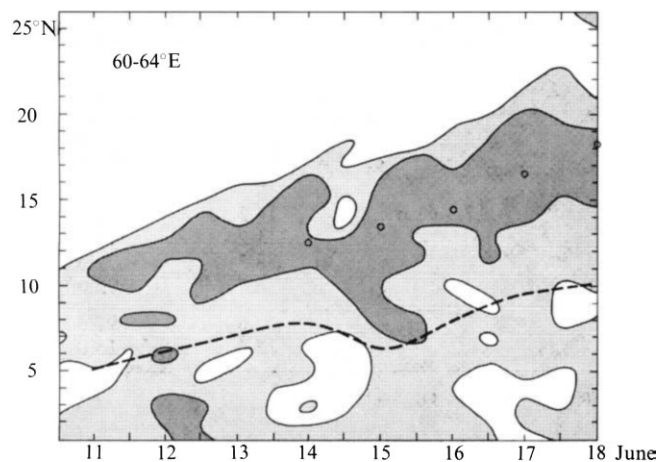


Fig. 2 Time-latitude section of rain frequency averaged over a strip from 60 to 64° E. The convention is the same as for Fig. 1.

with a large mass of mostly heavy rain in the north-central part of the Arabian Sea. The Somali jet bisected the rain area between the upper and lower masses, and the edge of the rain region lay 200–400 km east and south of the edge of the monsoon inversion.

Time changes in rain class frequency averaged across a longitudinal strip through the centre of the Arabian Sea (60–64° E) are shown in Fig. 2. Quite consistently, the heavier rain occurred on the north side of the rain area, to the left of the axis of the jet. The rain area spread steadily northwards, at ~150 km day⁻¹, or twice as fast as the northward movement of the jet axis.

It is expected from simple jet stream dynamics⁶ that over the Arabian Sea, rain would be heaviest to the right of the axis of the Somali jet. The departure observed here is apparently due to low level convergence occurring with the onset vortex, a cyclone which first appears on 14 June in the 850-mbar analyses of the atlas of Krishnamurti *et al.*⁷ The possibility has been suggested⁸ that the onset vortex received kinetic energy through baroclinic processes (as well as barotropic processes), perhaps even during its formation. Figures 1 and 2 tend to confirm this, by showing first, a concentration of rain—implying ascent and release of latent heat—in the north-west semicircle of the storm, which available temperatures and winds indicate was the warmer half between 850 and 700 mbar, and second, the presence of heavy rain at least 2 days before the vortex was detectable in low level winds.

For the Arabian Sea as a whole, the percentage coverage by rain of light, moderate or heavy rain classes is shown in Fig. 3.

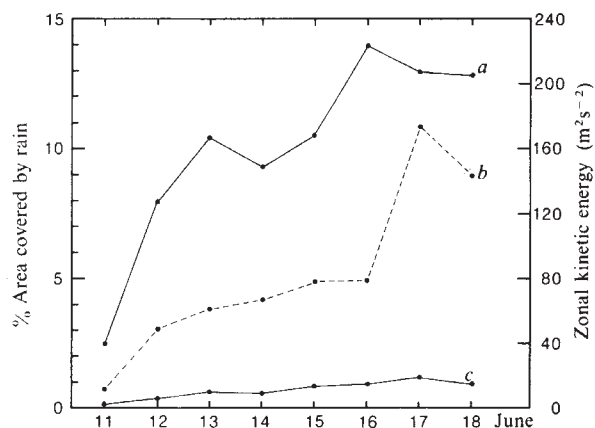


Fig. 3 Rain coverage over the Arabian Sea from 11 to 18 June. Each point is an average over the hours from 00.00 to 23.00 GMT. a, All rain classes. c, Heavy rain class only. b, Values of area-averaged zonal kinetic energy, from ref. 8.

The change over these eight days is remarkable: from 1% on 11 June to 14% on 18 June. The contribution of the heavy rain class to this growth is also shown in Fig. 3, from 0 to 1%. For heavy rain especially, the coverage curves closely parallel changes observed by Krishnamurti *et al.*⁸ in zonal kinetic energy averaged over the Arabian Sea, suggesting that the convective response of the atmosphere to accelerations of the low level flow was relatively fast.

We conclude that the technique described here can provide information on the changing pattern of rainfall over the Arabian Sea through the onset phase of the summer monsoon. The precise limits of its accuracy remain to be established.

During the 1979 onset a near-equatorial belt of rainfall spread northwards across the centre of the Arabian Sea. Heavy rains developed, in association with an onset vortex, on the north flank of the Somali jet and moved north as the jet moved north.

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Marine snow: major site of primary production in coastal waters

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Fragile aggregate particles, or 'marine snow', were collected by SCUBA from coastal surface waters using trace metal-free techniques, and their primary production (rates of photosynthesis by associated phytoplankton) was determined relative to corresponding water column production. Primary production associated with these particles was found to range from 11 to 58% of total production, indicating that large particles can have an important role as major sites of primary production in the marine environment. Our results suggest that the use of water bottles to collect samples for productivity measurements may not sample large particles adequately in certain conditions, and thus may lead to underestimates of primary production.

Macroscopic aggregates in the millimetre-centimetre size range are a common feature of the pelagic ecosystem. Compositionally, 'marine snow' is very complex and includes both organic and inorganic materials. Several origins for these amorphous aggregates have been suggested: adsorption of dissolved and colloidal carbon onto bubbles or other surfaces¹; mucus flocs derived from various zooplankton such as the houses of larvaceans² or mucus webs produced by pseudotoechomatous pteropods³; agglutination of fecal material or zooplankton remains⁴; or mucus colonies or mats formed from various types of phytoplankton.

Because of their fragile nature, the use of conventional collection techniques (such as nets or water bottles) has usually

resulted in disruption of these systems such that direct study was precluded⁵. Recently, techniques involving direct hand collection of marine snow in conjunction with SCUBA^{6,7} have provided relatively undisturbed samples of these particles which are amenable to laboratory analysis. Macroscopic aggregates serve as enrichment sites for chlorophyll⁶, various organic compounds such as proteins, carbohydrates and lipids⁷, microscale nutrient patches⁸ as microhabitats for various micro- and macro-organisms⁹, and as sites of metal concentration¹⁰. In addition, relative aggregate primary production has recently been determined from samples collected in the southern California Bight¹¹. Aggregate production values relative to total ranged from 0.1 to 9%. These are lower than the values we report here. This could reflect either regional differences in productivity, much lower (0.1–1.1 aggregates l⁻¹) particle concentrations, the use of distilled water to rinse productivity filters, or the fact that metal-free techniques were not used.

Recent work with sediment traps indicates that large particles represent an important fraction in terms of living biomass throughout the water column¹². These large particles are not typically collected using water bottles^{13,14}, and can only be collected using sediment traps (except in surface waters where some sampling by SCUBA is possible). Because of the large particle-living biomass relationship, and because water bottle collections do not adequately sample such large particles, we felt that a significant portion of phytoplankton primary production (given the biological and chemical associations) may be overlooked. We therefore collected large particles from the euphotic zone to determine their associated rates of primary production relative to 'ambient' (that is, containing no large particles) water from the same depth. Further, we compared these results with samples collected by standard procedures which involved water collection bottles.

Samples of marine snow were collected from two stations in the area of Monterey Bay, California in June and July 1981 from a depth of 10 m using SCUBA techniques similar to those devised for open-ocean blue-water diving^{5,6}. Aggregate abundance (Table 1) was determined using a 2.3-cm diameter loop mounted on a TSK flowmeter⁶. As is often the case with marine snow particles, particle size at a given station was remarkably homogenous. Aggregates from the June collections were all relatively large and well formed, while aggregates collected in July were smaller, more loosely consolidated, and appeared to be in an earlier state of development. Note that stringent anti-contamination methods were used during all phases of sample collection and manipulation^{10,15,16}, as significant reduction in primary production measurements can occur from metal

Table 1 Contribution of diver-collected marine snow aggregates to total primary production

Collection date	Mean no. (aggregates l ⁻¹)	Fractionated production (µg C l ⁻¹ h ⁻¹)			Niskin production* (µg C l ⁻¹ h ⁻¹)	Aggregate production (% total)	Niskin production (% total)
		Aggregate	Ambient	Total†			
June 1981	11.5 ± 3.1‡ n = 10	11.8	11.8	22.1	14.1 ± 4.7	53.4	63.8
		14.0	13.7	24.3		57.6	58.0
		11.4	8.4	21.7		52.5	65.0
		—§	7.4	—		—	—
		$\bar{X}$ 12.4	10.3	22.7		54.5	62.3
		± 1.4	2.9	1.4		2.7	3.7
July 1981	6.6 ± 2.8 n = 10	2.3	12.0	16.1	17.3 ± 1.6	14.3	107.
		1.8	12.2	15.6		11.2	111.
		8.5	16.1	22.3		38.3	77.6
		5.0	14.8	18.8		26.7	92.0
		$\bar{X}$ 4.4	13.8	18.2		22.6	97.0
		± 3.1	2.0	3.1		12.4	15.0

Total production is also compared with primary production values derived from Niskin bottle collections.

* Samples collected and processed using trace metal clean techniques¹⁷.

† Total indicates primary production derived from the sum of diver-collected aggregate and mean ambient (containing no aggregates) water fractions.

‡ All errors are ± 1 s.d.

§ Sample lost.

contaminants¹⁶. Aggregates were collected in 3–7 m lengths of acid-cleaned 9.5 mm diameter Bev-A-Line tubing. Before collections, the ends of the collection tubes were connected to form a continuous loop and placed into acid-cleaned plastic bags to avoid contamination. Polyethylene gloves were worn underwater while sampling and aggregate collection was directed into any perceivable current to avoid diver-related contamination. Sampling tubes were filled with either 15 (July collection) or 25 (June collection) aggregates per tube. In addition, at each station four tubes were filled with ambient seawater only, to assess the productivity of this fraction and also to correct for the inclusion of ambient seawater in the aggregate collection tubes. After collection, each sampling tube was reconnected, placed in its respective plastic bag, and returned to the ship for processing. Aboard ship, the contents of each tube (four tubes containing aggregates and ambient seawater and four tubes containing only ambient seawater) were emptied into 250 ml acid-cleaned polycarbonate flasks, injected with 10–20 μCi $\text{H}^{14}\text{CO}_3^-$ and incubated for 3 h in a seawater cooled deck-top incubator designed to simulate light levels at 10 m. Following the incubation period, samples were filtered and the filters counted by liquid scintillation to determine the amount of photosynthetically fixed carbon. The primary production procedure used followed clean techniques described in ref. 16.

At the same time as particle collection, water samples from each station were obtained from a depth of 10 m using standard 5-l Niskin bottles. These had been modified and acid cleaned to preclude metal contamination and mounted on non-metallic synthetic hydrolite¹⁷. Incubation, processing and counting of these samples followed the same procedure described above for the aggregate samples.

The results are presented in Table 1. It is obvious from the fractional production data that aggregate contribution to primary production can represent a significant portion of the total production. Thus, during the June sampling period when the aggregates appeared well formed and rather abundant (11.5 aggregates l^{-1}), >50% of the total production was associated with these particles. In contrast, during the July sampling period, the aggregates were less concentrated (6.6 aggregates l^{-1}), more loosely consolidated and probably in an earlier stage of development. Here, the aggregates contributed relatively less to total production with values ranging from 11 to 38%.

Another interesting aspect of this study, and a potentially serious problem, involves comparing total primary production derived from hand-collected samples with Niskin bottle collections (Table 1). It is apparent that, depending on the kinds of particles present in the water column, significant errors can result due to the failure of the bottles to sample the particle field adequately. For example, during the June sampling period, primary production calculations derived from Niskin bottle samples could have underestimated production by 40% (Niskin production = 62% of the total fractionated production). However, in July, when the particles were less consolidated, there was no significant difference between SCUBA and bottle-collected samples. Thus, experimental design flaws of this sort can not only lead to underestimates of this important parameter, but may also contribute to overall sampling variability.

For an accurate understanding of primary productivity processes in marine ecosystems, it is imperative that the role of large particles as well as efficient procedures for sampling these particles be established.

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Tulu Bor Tuff at Koobi Fora correlated with the Sidi Hakoma Tuff at Hadar

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The Hadar Formation of Ethiopia is renowned for its superb mammalian fossils, but there is still some doubt about the age of the Hadar faunas and their relations to faunas from other sites despite intense efforts to date the Hadar material^{1,2}. Here I report a stratigraphical tie between the Hadar Formation and formations in the Lake Turkana region, which helps to relate fossil faunas from the two areas.

The fossil site of Hadar is located along the lower reaches of the Awash River; the Shungura and Usno Formations are located in the lower Omo valley north of Lake Turkana, and the Koobi Fora Formation is located on the north-east side of that lake. The fossiliferous formations of the Lake Turkana region have many tuffs in common, and probably derive from a single depositional system dominated by the Omo River^{3,4}. The Awash River and the Omo River have a common drainage divide for a distance of ~100 km through the Guraghe Mountains northwards to Badda Rogghie. According to Mohr⁵, the Guraghe Mountains “are composed solely of silicic lavas and tuffs except for the summit line which is formed of superimposed massive trachyte”. The age of these lavas and tuffs is unknown, but if volcanoes in the general region of Mt Guraghe erupted during the Plio-Pleistocene, eruptive products might have found their way both to the lower Awash Valley, and into the Lake Turkana Basin.

A prominent tuff in the Koobi Fora Formation is the Tulu Bor Tuff, which is correlative with Tuff B of the Shungura Formation, and Tuff U-10 of the Usno Formation³. These tuffs are of normal polarity, and are thought to lie in the lowest normal part of the Gauss Normal Epoch, thus having an age between 3.17 and 3.42 Myr (ref. 6). Two minor chemical variants of the Tulu Bor Tuff have been recognized, which correspond to Tuff B- α and Tuff B- β of the Shungura Formation³. Walter¹ provides analyses of shards from the Sidi Hakoma

Table 1 Comparative analyses of the Sidi Hakoma Tuff and Tulu Bor Tuff microprobe analyses of major and minor elements (wt %)

	Sidi Hakoma range	Tuff* average	β -Tulu Bor Tuff†
SiO ₂	71.70–73.23	72.54	71.69
TiO ₂	0.12–0.24	0.16	0.14
Al ₂ O ₃	12.16–12.60	12.37	11.92
Fe ₂ O ₃ ‡	1.24–1.48	1.33	1.39
MgO	0.00–0.06	0.04	0.05
CaO	0.24–0.33	0.29	0.28

* From ref. 1. † From ref. 12. ‡ Total iron expressed as Fe₂O₃.

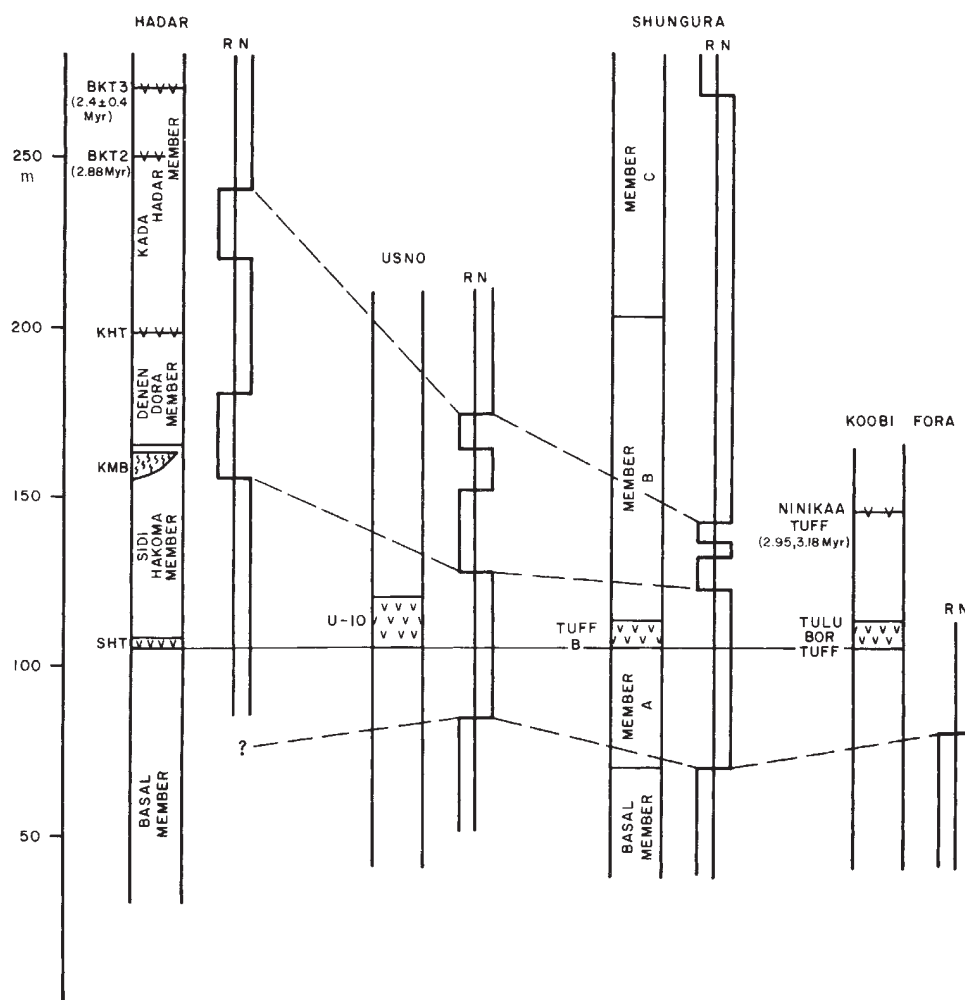


Fig. 1 Schematic partial stratigraphical columns for the Hadar, Usno, Koobi Fora and Shungura Formations. The magnetic polarity log for the Koobi Fora Formation is from ref. 13.

Tuff of the Hadar Formation, which are all similar in composition, and resemble analyses of the Tulu Bor Tuff (Table 1). The Sidi Hakoma Tuff most closely resembles Tuff B- β which is distinct in having lower CaO and TiO₂ contents than Tuff B- α . A glass separate from the Sidi Hakoma Tuff was prepared and analysed by methods described in ref. 3 (Tables 1 and 2). The remarkable compositional correspondence in trace elements confirms the identity of the two tuffs.

Therefore a direct lithostratigraphical tie exists between the Shungura, Usno, Koobi Fora, and Hadar formations, and as tuffs are isochronous horizons, a temporal line of correlation also extends to each of these localities. Schematic sections of

the localities under discussion are shown in Fig. 1, where it is seen that the record of magnetic polarity transitions is in good agreement amongst the sites, although thicknesses vary from one locality to another. Suid fossils from the Hadar Formation and from formations in the Lake Turkana Basin have already led Cooke⁷, and Harris and White⁸ to suggest correlations similar to those proposed here (not as misquoted in ref. 9, p. 207).

The two reversed magnetozones above the Sidi Hakoma Tuff, and their apparent correlatives in the Shungura and Usno Formations, have been independently identified as the Mammoth and Kaena Events^{6,10}. Such identifications are consistent with all K/Ar and fission-track ages from the Hadar Formation with the exception of the 3.6-Myr age on the Type B Kadada Moumou Basalt (KMB). The mean of all ages on the Type A KMB is 3.01 Myr, which when corrected upward by 3.5% (see statement in ref. 11) would be 3.12 Myr, in concordance with its reversed polarity. These identifications are also in accord with K/Ar dates on tuffs from the Lake Turkana Basin except for the dates on Tuff B-10 of the Shungura Formation, which are apparently too old (see ref. 6).

As most of the fossil material at Hadar derives from levels between the Sidi Hakoma Tuff and BKT-2 (see Fig. 1), which is only a few metres above the top of the Kaena Event, these faunas must correlate with faunas from the lower part of Member B of the Shungura Formation, and with those of the Usno Formation. It is likely that these faunas all lie between 3.3 and 2.8 Myr in age. This chronology is consistent with the palaeomagnetic record at Hadar and in the Lake Turkana Basin, with the suid evidence, and with all radiometric age determinations except those noted above. The mean sediment accumulation rate for the interval between the Sidi Hakoma Tuff and BKT-2 is ~ 35 cm kyr⁻¹, which is also reasonable. The ages on

Table 2 X-ray fluorescence analyses of minor and trace elements (values in p.p.m. except where noted)

	SHT-106 [†]	80-177 [‡]	Range β -Tulu Bor Tuff (16 samples)
CaO (%)	0.32	0.31	0.28–0.36
Fe ₂ O ₃ * (%)	1.56	1.58	1.45–1.77
Ba	144	134	112–256
Mn	381	392	351–435
Nb	84	85	78–86
Rb	110	111	108–126
Sr	8	5	5–16
Ti	884	987	877–1147
Y	61	62	57–65
Zn	86	77	72–100
Zr	369	375	351–453

* Total iron expressed as Fe₂O₃.

[†] Sidi Hakoma Tuff.

[‡] β -Tulu Bor Tuff, Area 117, Koobi Fora.

the Type B KMB are evidently too old, but this discrepancy has not yet been explained.

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Faunal age of the Usno, Shungura B and Hadar Formations, Ethiopia

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Recent work by Brown¹, correlating the Sidi Hakoma (or Sidiha Koma²) Tuff at Hadar with Tuffs B and U-10 of the Shungura and Usno Formations, respectively, reverses a recent finding³ indicating an age of the Hadar Formation fauna of 2.9 to greater than 3.6 Myr BP. The difference in these determinations is 0.4 Myr, a significant period of evolutionary time. We compare the faunas from Shungura Unit B10, Usno and Hadar and discuss the implications for interpretation of the early hominid fossil record.

The fauna from Member B of the Shungura Formation derives primarily from the uppermost units, particularly Unit B10, superjacent to the B10 Tuff dated at 3.03 ± 0.07 Myr BP⁴. The faunal sample from White Sands and Brown Sands localities of the Usno Formation, which has been correlated with lower Member B (Unit B2), is situated stratigraphically near Tuff U10 dated at 3.05 ± 0.30 Myr BP⁴ and above the basalt of U1 dated at 3.39 ± 0.23 Myr BP⁴. The Shungura B10 fauna occurs above the top of the Kaena Event (2.92 Myr BP), and the Usno fauna straddles the bottom of the Mammoth Event (3.15 Myr BP) but is younger than the Gilbert–Gauss boundary at 3.4 Myr BP⁵. Using the K/Ar and palaeomagnetic determinations to estimate best-fit ages of 2.92 and 3.15 Myr BP for B10 and Usno/Lower B faunas, respectively, it is clear that they are separated by ~0.2 Myr. Considerations of the age of the Shungura Member B fauna therefore must include a realization that there is significant time difference between upper (B10) and lower (B2) portions of the member, the latter equivalent in time to Usno.

Table 1 gives large mammal species lists for Shungura B10, the Usno Formation and the Hadar Formation, compiled from published sources^{6–13}, the Omo faunal catalogue and our own interpretations, except where indicated. Table 2 summarizes results from six faunal similarity coefficient calculations. By any method the Usno and Hadar faunas are more similar than are Shungura B10 and Hadar faunas, thus indicating a probable age for Hadar nearer to 3.15 Myr BP than to 2.92 Myr BP.

Comparison of similarity coefficients based on presence or absence of taxa at Hadar with faunas in the range 3.2–4.0 Myr BP, such as at Laetoli, Kanapoi, Mursi and Ekora, is hindered

by the small and not fully studied or published faunal inventories from the latter sites. However, by a consideration of evolutionary stages of single taxa, particularly in this case, those at Hadar not shared with Usno, biostratigraphical evidence can be obtained. The species not shared are: *Parapapio* cf. *jonesi*, *Theropithecus darti*, *Canis* aff. *mesomelas*, *Pachycrocuta* aff. *perrieri*, *Loxodonta adaurora*, *Elephas recki brumpti* (ex-'Stage 1'), *Ceratotherium* cf. *praecox*, *Kolpochoerus afarensis*, *Giraffa stillei*, *Praedamalis deturi*, and *Madoqua ?avifluminis*. Unshared generic identifications at Hadar of potential biostratigraphical importance are *Mammuthus* sp., *Ugandax* sp. nov., *?Pelorovis* sp. and *Ovibovini* sp. aff. *'Bos' makapaani*.

The first four species listed and *Elephas recki* occur in the South African middle to late Pliocene site of Makapansgat¹⁴, as do three other shared Hadar-Usno species. Unshared *'Bos' makapaani* from Buffalo Cave, Makapansgat may be of similar age. *E. recki* and *?Pelorovis* sp. are Hadar taxa not shared with Usno but present in the later Shungura B10 fauna. *Hipparion afarensis* is more primitive than *H. ethiopicum*, which first occurs in Shungura Formation Member C, but is more advanced (and apparently later in time) than *H. sitifense* found in both Shungura B10 and Usno faunas¹⁶. *Loxodonta adaurora* and *Ceratotherium praecox* occur in the Omo Mursi Formation, at ~4 Myr BP⁷. Cooke⁹ has discussed *Kolpochoerus afarensis* as ancestral to, but especially as seen in the larger specimens, very similar to *K. limnetes*, found in Usno and B10 assemblages. *Giraffa stillei*, although known from Laetoli, is considered by Churcher¹⁷ to range from the "early Pliocene to early middle Pleistocene" and it may prove synonymous with *G. gracilis*, present at Usno and Shungura B10. Gentry¹⁰ considers *Praedamalis deturi* suggestive of "an older age than Shungura upper Member B" and Hadar *Madoqua ?avifluminis* similar to specimens from the Ndolanya Beds at Laetoli. The Ndolanya Beds are dated to between 2.41 ± 0.12 and 3.49 ± 0.12 Myr BP¹⁸, in the same age range as the Usno Formation. The genus *Mammuthus*, with three recognized species, ranges in Africa from the Pliocene to the early Pleistocene¹⁹. *Ugandax* sp. is "no later than Shungura upper B and probably earlier"¹¹.

The faunal similarities with Makapansgat, with Shungura B10 and the presence of *Hipparion afarensis* are suggestive of an age younger than the Usno Formation, while *Loxodonta adaurora*, *Ceratotherium praecox*, *Kolpochoerus afarensis* and perhaps *Giraffa stillei* suggest an older age. The bulk of the diagnostic fauna, however, is consistent with an Usno/Shungura lower B age for the Hadar fauna.

This finding has several implications for interpretation of the fossil hominids from the Hadar Formation. Because the fossil-bearing Hadar Formation is constrained between SHT (~3.2 Myr BP, based on ref. 1) on the lower end and BKT-2 (2.88 ± 0.08)³ and the top of the Kaena Event (~2.92 Myr BP) on the upper end, the hominids are relatively closely spaced in time. There are not more than some 300,000 yr between hominids at the top and bottom of the sequence.

The temporal correlation of Pre-Kadada Moumou (or Kada Damoumou²) Basalt Hadar hominids with Laetoli hominids³ (dated now between 3.49 ± 0.03 Myr BP and 3.76 ± 0.03 Myr BP¹⁷) is called into question. There may be between 0.3 and 0.9 Myr difference in time between the two parts of the type-series of *Australopithecus afarensis*²⁰.

The similarity in time between Hadar hominids and those from Makapansgat may be closer than suspected²¹. Not only does Hadar now appear later in time, but several indications seem to place Makapansgat earlier. Cooke¹⁴ records from this site *Notochoerus capensis*, also known from the 'Kubi Algi' Formation and possibly Kanapoi²², both apparently near 4 Myr BP, and *Potamochoeroides shawi*. The latter is termed *Metridiochoerus andrewsi* (I) by Harris and White²³ but Cooke²² regards it as a South African endemic Pliocene suid not particularly useful for biostratigraphical correlation. Cooke¹⁴ does not recognize at Makapansgat the typical *Metridiochoerus andrewsi* which is found in Shungura Member G (not as reported in ref. 21). An additional indicator for the 'old' age at

Table 1 Species lists of the large mammals from Hadar, Usno and Shungura B10

Hadar Formation	Omo Shungura B-10	Omo Usno Formation
<i>Parapapio</i> cf. <i>jonesi</i> *	—	—
<i>Theropithecus darti</i> +	<i>Theropithecus brumpti</i> +	<i>Theropithecus</i> sp.
—	<i>Cercopithecus</i> sp.	<i>Cercopithecus</i> sp.
<i>Rhinocolobus</i> cf. <i>turkanaensis</i> *	<i>Rhinocolobus turkanaensis</i> *	<i>Rhinocolobus turkanaensis</i> *
—	<i>Paracolobus mutiwa</i>	—
—	<i>Papio</i> sp.+	<i>Papio</i> sp.+
<i>Galerella</i> sp.	—	—
—	<i>Helogale</i> sp.	—
<i>Herpestes</i> sp.	—	—
<i>Viverra</i> (<i>Civettictis</i>) sp.	<i>Viverra</i> (<i>Civettictis</i>) sp.	—
—	<i>Viverra leakeyi</i>	<i>Viverra</i> cf. <i>leakeyi</i>
—	<i>Pseudocivetta ingens</i>	—
aff. ? <i>Poecilogle</i> sp.	—	—
—	—	<i>Lutra</i> sp.
<i>Enhydriodon</i> sp. nov. A	<i>Enhydriodon</i> sp. nov. B	<i>Enhydriodon</i> sp. nov. B
—	—	—
<i>Canis</i> aff. <i>mesomelas</i>	—	—
<i>Pachycrocuta</i> aff. <i>perrieri</i>	—	—
cf. <i>Crocuta</i> sp.	—	—
<i>Percrocuta</i> sp.	—	<i>Percrocuta</i> sp.
—	<i>Hyaena</i> aff. <i>makapani</i>	<i>Hyaena</i> aff. <i>makapani</i>
<i>Megantereon</i> sp.	? <i>Megantereon</i> sp.	<i>Megantereon</i> sp.
<i>Homotherium</i> sp.	<i>Homotherium</i> sp.	<i>Homotherium</i> sp.
<i>Dinofelis</i> sp.	<i>Dinofelis</i> sp.	<i>Dinofelis</i> sp.
<i>Panthera</i> sp.	<i>Panthera</i> sp.	<i>Panthera</i> sp.
<i>Felis</i> sp.	<i>Felis</i> (<i>Lynx</i>) <i>caracal</i>	—
<i>Deinotherium bozasi</i>	<i>Deinotherium bozasi</i>	<i>Deinotherium bozasi</i>
<i>Loxodonta adaurora</i>	—	—
<i>Elephas ekorensis</i>	—	<i>Elephas ekorensis</i>
<i>Elephas recki brumpti</i>	<i>Elephas recki brumpti</i>	—
<i>Mammuthus</i> sp.	—	—
<i>Hipparion afarense</i>	—	—
—	<i>Hipparion sitiense</i>	<i>Hipparion sitiense</i>
<i>Ceratotherium</i> cf. <i>praecox</i>	—	—
<i>Ceratotherium simum</i>	<i>Ceratotherium simum</i>	<i>Ceratotherium simum</i>
<i>Diceros bicornis</i>	<i>Diceros bicornis</i>	<i>Diceros bicornis</i>
<i>Kolpochoerus afarensis</i>	—	—
—	<i>Kolpochoerus limnetes</i>	<i>Kolpochoerus limnetes</i>
—	<i>Metridiochoerus jacksoni</i>	—
<i>Notochoerus euilus</i>	<i>Notochoerus euilus</i>	<i>Notochoerus euilus</i>
<i>Nyanzachoerus kanamensis</i>	<i>Nyanzachoerus kanamensis</i>	<i>Nyanzachoerus kanamensis</i>
<i>Hexaprotodon</i> aff. <i>protamphibius</i>	—	<i>Hexaprotodon</i> aff. <i>protamphibius</i>
<i>turkanaensis</i> (= "Afar sp. A")	—	<i>turkanaensis</i> (= "Afar sp. A")
—	<i>Hexaprotodon shungurensis</i>	—
<i>Hexaprotodon</i> sp. (= "Afar sp. B")	—	—
<i>Sivatherium maurusium</i>	<i>Sivatherium maurusium</i>	<i>Sivatherium maurusium</i>
—	<i>Giraffa gracilis</i>	<i>Giraffa gracilis</i>
<i>Giraffa stillei</i> (?aff. <i>gracilis</i>)	—	—
—	<i>Giraffa "pygmaeus"</i>	—
<i>Giraffa jumae</i>	<i>Giraffa jumae</i>	? <i>Giraffa jumae</i>
<i>Tragelaphus nakuae</i>	<i>Tragelaphus nakuae</i>	<i>Tragelaphus nakuae</i>
<i>Tragelaphus</i> sp. nov.	—	—
—	<i>Tragelaphus gaudryi</i>	—
—	<i>Taurotragus</i> sp.	—
<i>Ugandax</i> sp. nov.	—	—
—	<i>Syncerus</i> sp.	—
? <i>Pelorovis</i> sp.	<i>Pelorovis</i> sp.	—
<i>Praedamalis deturi</i>	—	—
<i>Kobus</i> sp. A	—	—
<i>Kobus</i> sp. B	—	—
<i>Kobus</i> sp. C	—	—
—	<i>Kobus kob</i>	—
—	<i>Kobus ancyroscera</i>	—
—	<i>Redunca</i> sp.	—
? <i>Damalops</i> sp.	—	—
—	<i>Megalotragus ?kattwinkeli</i>	—
—	? <i>Oreonagor</i> sp.	—
<i>Aepyceros</i> sp.	<i>Aepyceros</i> sp. nov.	<i>Aepyceros</i> sp. nov.
—	<i>Antidorcas recki</i>	—
<i>Gazella</i> sp.	—	<i>Gazella</i> sp.
<i>Madoqua ?avifluminis</i>	—	—
? <i>Raphicerus</i> sp.	—	—
<i>Ovibovini</i> sp. (aff. " <i>Bos</i> " <i>makapaani</i>)	—	—

Compiled from refs 6–13. *, Identification by E. Delson, +, identification by G. G. Eck. Hominid taxa and identifications above the subfamilial or tribal level are not included. For the purposes of calculating the similarity coefficients in Table 2, qualified species identifications (cf., aff. and ?) have been counted as occurrences. Identifications to genus only are not counted in calculations.

Table 2 Faunal similarity coefficients among Hadar, Usno and Shungura B10 faunas

Faunal comparison	Hadar-Usno	Hadar-Shungura B10	Usno-Shungura B10
Simpson (C/N_1)	0.61	0.35	0.88
Jaccard ($\frac{C}{N_a + N_b - C}$)	0.28	0.20	0.53
Kulczynski ($\frac{C(N_a + N_b)}{2N_a N_b}$)	0.48	0.33	0.73
Burt-Pilot ($\frac{2C}{N_a + N_b}$)	0.44	0.33	0.69
Otsuka ($\frac{C}{\sqrt{N_a N_b}}$)	0.46	0.33	0.71
Braun-Blanquet ($\frac{C}{N_2}$)	0.35	0.32	0.57

Formulae are from ref. 26. N_a and N_b are observed number of species in the two assemblages, C is the number of species in common, N_1 is the smaller and N_2 the larger of N_a and N_b .

Makapansgat is a recent report by Lehmann and Thomas²⁴ that the Makapansgat bovid, *Redunca* aff. *darti* also occurs in an upper level of the basal Pliocene Sahabi Formation, Libya. *Nyanzachoerus kanamensis*, a species found at Hadar and Usno, occurs in this same level at Sahabi²⁵. Thus it is far from clear that Makapansgat *Australopithecus africanus* post-dates Hadar *A. afarensis*. Hadar and Makapansgat may be broadly equivalent in faunal age to the Usno Formation, although

Makapansgat probably ranges later in time.

Because of the poor knowledge of African faunas in the 3.2–4.0 Myr BP time range, it is difficult to reject categorically a pre-Usno age for Hadar. Nevertheless, our results are consistent with the age suggested by Brown¹ for the SH Tuff at Hadar.

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Evidence that seals may use echolocation

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Harbour seals (*Phoca vitulina*), like many phocid species, forage to some extent at night¹. Although nocturnal mammals typically rely on non-visual sensory channels, seals have adapted to low light levels by sharpening their visual sense². Although enhanced visual sensitivity would be functional in clear water, many species inhabit turbid coastal or estuarine regions where vision is sometimes of limited use even in daylight. Here we present experimental evidence in support of the contention that when visual cues are not available, seals use echolocation. Our results help to explain why previous attempts to demonstrate sonar abilities in pinnipeds have been unsuccessful.

Circumstantial evidence suggests that pinnipeds use some form of echolocation. Some species produce click vocalizations similar to the signals of animals that are known to have a sonar system³. Harbour seals click more frequently in the dark⁴ and there are numerous reports of blind seals functioning sufficiently well to raise a pup⁵. However, all experimental evidence has

been negative. Evans and Haugan⁶ reported that although a sea lion (*Zalophus californianus*) was able to retrieve rings at night, no vocalizations were recorded, and the animal refused to perform when blindfolded. Schusterman⁷ tried to train *Zalophus* to discriminate between visually identical plexiglass disks, one filled with air, the other solid so that each disk would return a different echo to the animal. After over 2,000 trials the sea lion was unable to distinguish reliably between the targets, even though click trains were recorded on most trials. Scronce and Ridgway⁸ trained a blindfolded grey seal (*Halichoerus grypus*) to retrieve a ring, and later presented the seal with the task of discriminating between two differently sized styrofoam surfaces. These authors concluded that the seal was not using sonar because few clicks were recorded during retrieval, and because the animal, having produced no vocalizations, was unable to learn the styrofoam discrimination.

Our recordings in the field⁴ and from captive animals indicate that harbour seals produce at least two types of signals. The first is a brief, rapid-onset pulse which is emitted either as a single click or as a doublet (see Fig. 1a). Because of its fast onset, the click spans the entire audio spectrum, however most of the energy lies near 7.5 kHz. The second signal consists of a train of these clicks emitted in rapid succession such that the interval between each one determines the overall pitch of the burst (see Fig. 1b).

At Miquelon (45° 45' N, 56° 14' W) where there is a breeding colony of *P. vitulina*, we found that both vocalizations were

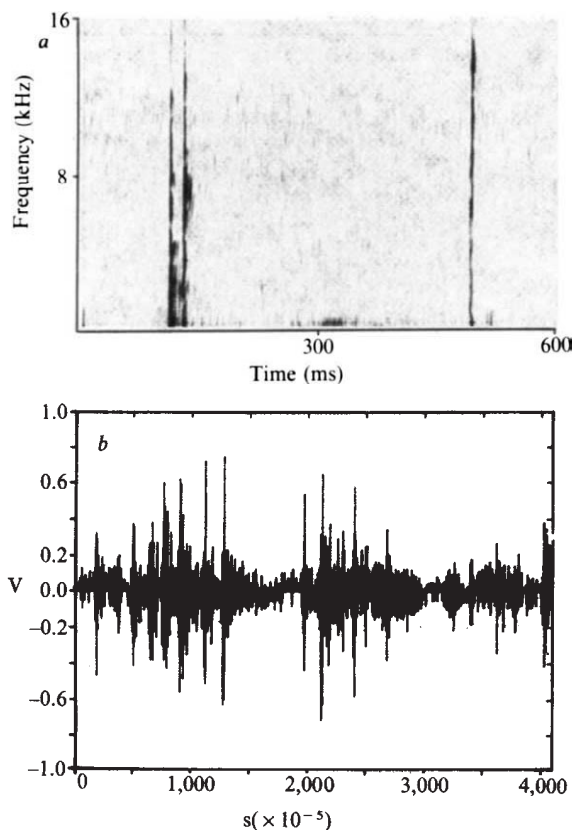


Fig. 1 *a*, A sonogram of a single and a doublet click made by the seal during discrimination training. *b*, Waveform of a train of clicks recorded from a breeding colony of harbour seals at Miquelon.

more frequent at night. However, before the present experiment, our captive harbour seals would only click when given live fish in total darkness. In early attempts to use Schusterman's procedure⁷ with a harbour seal, we found that the animal would not click in the presence of the disks, perhaps because it had no understanding of the fact that echoes might be of assistance. We then modified the method to enhance the likelihood that the seal would use sonar to solve a similar discrimination problem.

The subject of the experiment was a 7-yr-old male harbour seal who has lived in captivity since it was collected as a weaned pup. The apparatus consisted of two rings made of hollow black plastic tubing, 4 cm in cross-section. The inner diameter of each ring was 9 cm, and the outer diameter 17 cm. One ring was internally ballasted with 600 g of lead; the other contained 101 g of lead and was filled with water. Both rings were negatively buoyant and during the second phase of the experiment were suspended 0.5 m apart in the water by 1 m-long strings such that the ballast lay at the bottom of each ring. The string of the air-filled ring had a small loop at its free end so that we could distinguish between each ring during testing.

The study was done in an outdoor 7.5-m diameter tank above ground, constructed from 6.5-cm white pine. It was filled with seawater to 1.2 m depth. For certain parts of the experiment a black vinyl tarpaulin, suspended over a superstructure ~1 m above the tank, was draped to the ground so that we could work under it.

A Gould model CH-17U hydrophone was used to monitor vocalizations. During the second phase of the experiment it was positioned at the same depth as the rings, ~1.5 m to the side of the nearest ring. Signals were recorded with a Sony model TC-D5 cassette tape recorder, and a Racal Store 4 instrumentation recorder was used to determine any ultrasonic components.

The seal was trained to retrieve the air-filled ring to earn a piece of herring. The ring was slipped into the water on one side of the tank, while the seal waited, with its head out of the water, at a station on the opposite side until one of us said "fetch". The seal then located the ring, put its snout through the centre and in this way carried it back to the experimenter and tossed it over the side. Once the animal was able to do this, it was required to do so at night when the tarpaulin was in place to eliminate ambient light. We recorded the latency between the "fetch" call and the moment when the seal returned with the ring.

After seven sessions of 10 trials each, the seal was then presented with two rings, and required to retrieve the air-filled one. In this case, one experimenter held the free end of the strings to suspend the rings ~0.6 m below the water surface, ~0.8 m away from the tank wall. When the seal chose the air-filled ring, it was released and the animal brought it across the tank to the other experimenter who rewarded it with a piece of fish. If the water-filled ring was chosen it was pulled off the seal's snout, and both rings removed from the water, at which time the seal was given a 30-s rest period as 'punishment'. At least 20 trials were run in each session, and the position of the correct ring (left as opposed to right) was varied at random. After seven sessions run in the dark, the training was continued in daylight as the rings were visually identical. When the seal learned the discrimination, the air-filled ring was ballasted at 101 g and filled with water, and five additional sessions were run. Vocalizations were monitored during all sessions with the Sony recorder, and on three occasions with the Store 4.

During the first phase of the study, when the seal was required to find one ring in the dark, the animal returned it to the experimenter in, on average, 34 s (s.d. = 50.47), although the time it took to locate the ring was slightly less than this. Single and doublet clicks were emitted throughout each session, and click trains were recorded for ~40% of the trials.

During the first session of discrimination training when the seal had to identify the air-filled ring, the correct ring was chosen on 63% of the trials. This level of performance improved until the seal was able to respond correctly on at least 75% of the trials ($t = 2.34$, $P < 0.05$ for trials 1–13 compared with 14–26), the best discrimination ratio being 0.85. The only time the animal responded at less than 50% was in a few sessions just after we switched to training in daylight (see Fig. 2).

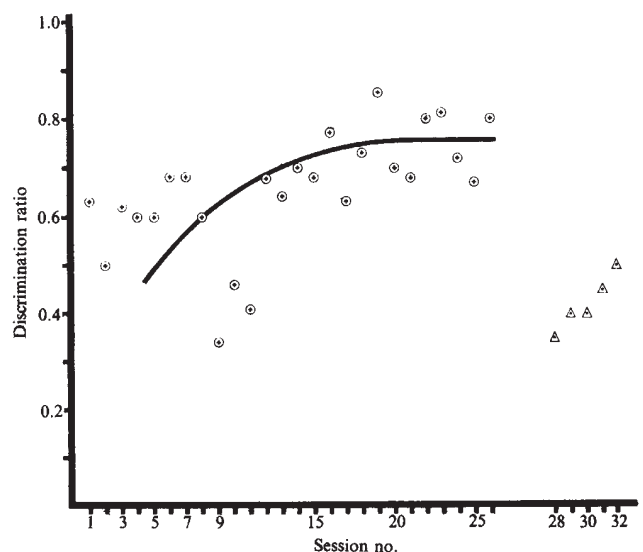


Fig. 2 Performance of the seal during discrimination training (⊕) and after both rings were filled with water (Δ). The discrimination ratio is the percentage of trials in which the seal chose the correct ring. Training occurred in darkness for the first seven sessions, and thereafter in daylight. The line was fitted by eye.

After 26 training sessions, both rings were filled with water and the seal's performance fell to chance levels ($\bar{x} = 0.42$, s.d. = 0.06, $t = 6.82$, $P < 0.05$ for control trials compared with the last five discrimination trials; see Fig. 2).

The only vocalizations that were consistently produced during discrimination training were very faint single or doublet clicks. No click trains such as those emitted during the location task were recorded. There was no evidence of an ultrasonic component to either type of signal.

The following results suggest that the seal was using echolocation: (1) it was able to locate a ring in total darkness; (2) it could discriminate between two objects that were visually identical but which differed in acoustic impedance; (3) the seal produced click vocalizations during the performance of both tasks; and (4) when the same rings were both filled with water, the animal was no longer able to distinguish between them even though it clicked while attempting to retrieve the correct one.

In our pilot study, we were unable to train a harbour seal using the same procedure as that used by Schusterman⁷. We found, as did Scronce and Ridgway⁸ and Evans and Haugan⁶, that the seal did not click during testing. This was perhaps not surprising as there was no reason to expect the animal to realize the task could be solved by using sonar. We therefore introduced the intermediate step of requiring the animal to first locate one ring in the dark, a situation in which it might be predisposed to using echolocation. For similar reasons we ran the first few sessions of discrimination training in the dark. Throughout the seal's early training sessions, it chose the air-filled ring correctly on about two-thirds of the trials, and only scored < 0.50 after we switched to daylight testing. It is tempting to speculate that in the dark when the visual similarity of the rings was not apparent, the seal favoured the air-filled one because it was most salient, returning the loudest echo. However, the discrimination ratios in the seven sessions in the dark and for the first seven in daylight are not statistically different ($t = 0.79$, $P > 0.05$), and it is therefore impossible to determine whether the change in the seal's behaviour was a function of the rings being visible or a reflection of performance instability during early training. Similarly, the increase in the discrimination ratio from 0.35 to 0.50 over the sessions when the seal was presented with two water-filled rings is not statistically significant ($\chi^2 = 1.32$; $P > 0.05$).

The seal seemed to find the discrimination task difficult; perhaps its sonar system is not sophisticated enough to easily make distinctions like the one we devised. Most proficient echolocators operate primarily in the ultrasonic range as the higher the carrier frequency and/or pulse repetition rate, the better the resolution of the system. Certainly, an echolocator having a low frequency signal would be at a disadvantage in an enclosed tank in which reverberant surfaces are close, and ambient noise high. Quiet individual or doublet clicks emitted after perceptible delays might have been the seal's solution to the problems of reverberation, especially during discrimination training when the rings were close to the tank wall.

Although our experiment provides evidence of echolocation, a study of this type cannot eliminate unequivocally the possibility that some cue of which we were unaware, may have somehow assisted the seal. Norris³ cautioned that the only way to unequivocally demonstrate echolocation is by blocking the animal's sound reception or emission during discrimination performances. To date, no marine mammal has willingly participated in such a procedure and as Norris goes on to admit, the general acceptance of dolphin sonar abilities rests on "less than perfect experiments". If some method of performing the critical test could be devised which would ensure that the animal was not disrupted in a general way, all doubts about echolocation in pinnipeds could be allayed once and for all.

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Isolation of infective tomato bushy stunt virus after passage through the human alimentary tract

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Research on plant virus vectors has shown that almost all types of organisms feeding on or parasitizing infected plants, including sucking and biting insects, mites, nematodes and chytrid fungi, can act as specific vectors. Surprisingly, however, natural vectors have not been found for some of the most infectious of the plant viruses such as tomato bushy stunt virus (TBSV) and tobacco mosaic virus (TMV). Here we report that when purified TBSV was consumed by human volunteers, it subsequently occurred in their faeces and could be detected by mechanical inoculation of faecal extracts to the leaves of an indicator plant (*Chenopodium quinoa*) in which the virus causes countable, discrete lesions. Thus, the virus remained stable on passage through the alimentary tract. The significance of man as a carrier, able to disseminate the virus widely within the environment, is discussed in relation to the epidemiology of the disease. To our knowledge, this is the first demonstration of an 'alimentary-resistant' plant virus.

TBSV was first isolated¹ from tomato (*Lycopersicon esculentum*) crops in Ireland in 1935 and was later found in tomato crops in England². The virus occurs in nature as a number of different pathogenic but serologically-related strains infecting a variety of crops and other plant species³ including tomato, pepper, globe artichoke, cherry, hop, grapevine, *Pelargonium* and *Petunia*. Recently, it has caused economically important tomato diseases in Morocco⁴ and Tunisia (C. Cherif, personal communication) because diseased plants are stunted and the 'mottled' fruit is unacceptable for export. The distribution of TBSV includes western and central Europe, North Africa, Argentina, Canada and Mexico.

TBSV is a single-stranded RNA-containing virus with isometric particles ~30 nm in diameter. In some experimental hosts (for example, *Nicotiana glauca*) it may reach a concentration of ~200 mg per kg of infected leaf. It is readily transmissible by mechanical inoculation and infective leaf

Table 1 Virus recovery from faeces of three volunteers 1–5 days after ingesting TBSV

Treatment	Volunteer	Dilution of sample	Infectivity* of faecal samples on day:					
			0†	1	2	3	4	5
TBSV in distilled water	T.F.	0 1/10	0 0	8 5	5 2	0 0	0 0	0 0
	J.F.	0 1/10	0 0	>100 92	28 16	3 2	1 1	0 0
	J.G.	0 1/10	0 0	>100 >100	19 10	32 23	0 0	0 0
Distilled water	M.J.	0 1/10	0 0	0 0	0 0	0 0	0 0	0 0
	E.B.							
	A.C.							

Three volunteers consumed 10 mg purified TBSV in 50 ml distilled water and three volunteers (controls) received water alone. TBSV was purified from systemically infected *N. clelandii* leaves by *n*-butanol (8.5% v/v) clarification of leaf homogenates in 0.5 M potassium phosphate buffer (pH 7.5) followed by differential centrifugation and density gradient centrifugation on 10–40% (w/v) sucrose columns in 0.01 M phosphate buffer (pH 7.5). Virus zones containing TBSV were removed, diluted in distilled water and ultracentrifuged. Virus pellets were resuspended in distilled water and stored at -18°C . Faeces were stored frozen. Samples (2–3 g) were homogenized in 0.05 M phosphate buffer pH 7.2 containing $200\text{ }\mu\text{g ml}^{-1}$ penicillin, $200\text{ }\mu\text{g ml}^{-1}$ streptomycin and $10\text{ }\mu\text{g ml}^{-1}$ amphotericin in sodium deoxycholate (Fungizone). After centrifugation at 5,000g for 15 min to remove solids, the supernatant was centrifuged for 30 min at 100,000g. Faecal pellets were resuspended for 4 h in 1.0 ml 0.005 M phosphate buffer pH 7.5 (dilution 0) and aliquots diluted 1/10 in the same buffer. Samples (coded) were manually inoculated to carborundum-dusted *C. quinoa* leaves. Resultant TBSV lesions were counted 5 days after inoculation and recorded before the code was known.

* Mean no. of TBSV lesions in four *C. quinoa* leaves.

† Virus was consumed a few hours after the first collection of faecal samples on day 0.

extracts retain infectivity after freezing for several years⁵. The thermal inactivation point is between 80 and 90 $^{\circ}\text{C}$.

TBSV is soil-borne⁶ and, although most other soil-borne viruses have nematode or fungus vectors, no vector of TBSV is known at present^{3,7}. Studies, begun in 1980, on the occurrence of plant viruses in English rivers⁸ have isolated TBSV from the rivers Thames and Cam, from tributaries of the rivers Avon (Warwickshire) and Trent and also from Lake Esthwaite (Cumbria). Present investigations on the origin of TBSV in rivers have demonstrated its occurrence in consignments of symptomless tomatoes imported from Morocco to Birmingham in 1982 and in tomato plants growing extraneously in sludge at sewage works in Warwickshire and Leicestershire⁹. In an attempt to explain its presence in river water and in the tomato plants growing in sludge, we have postulated that the virus remained infective after human consumption of TBSV-infected tomato fruit and that it was then transported to rivers after treatment of infective faeces at sewage works.

An *in vitro* test showed that the infectivity of TBSV (isoelectric point, pH 4.1) was retained after storage at pH 3.0, a value similar to that of many samples of gastric juice. An homogenate of TBSV-infected *N. clelandii* leaves in distilled water (1 g per 2 ml) was clarified by centrifugation at 5,000g for 10 min and aliquots of the infective supernatant fluid adjusted with 10% HCl to pH 3.0, 3.5 and 4.0. An unadjusted aliquot (pH 6.5) was used as a control. After incubation for 1 or 3 h at 25 $^{\circ}\text{C}$, the samples were neutralized (pH 7.0) with NaOH, stored at 4 $^{\circ}\text{C}$ for 5–6 h to allow virus resuspension and 10-fold dilutions of the samples assayed for infectivity by mechanical inoculation of *C. quinoa* leaves. Based on the numbers of lesions formed in the leaves after 5 days, no differences in the infectivity end points were found between the acid-treated and control samples.

The *in vivo* experiment was as follows. Six adult volunteers were randomly divided into two groups. Each of three volun-

teers (T.F., J.F. and J.G.) consumed 10 mg of purified TBSV in 50 ml distilled water and three volunteers (M.J., E.B. and A.C.) received water alone. Each volunteer consented not to eat tomatoes for 7 days before the start of the experiment. Faeces was collected from each volunteer 1 day before and daily for 5 days after consuming the test liquids. After storage at -18°C , the faecal collections were sampled and assayed for TBSV infectivity (Table 1). TBSV was detected in faeces of each of the volunteers receiving virus but not in those from the control volunteers. For individuals receiving virus, the infectivity was similar and high in faecal extracts of two volunteers (J.F. and J.G.) but it was relatively low in the case of the third volunteer (T.F.). The reason for this is unknown, but may be due to errors in faecal sampling.

The result demonstrates the remarkable stability of TBSV in that it retained its infectivity after ingestion and successive exposure at 37 $^{\circ}\text{C}$ to the enzymes of the alimentary tract, bile salts and wide fluctuations in pH (~ 3.0 –8.8).

Retrospectively, the chances of plant viruses surviving passage through the alimentary tract have usually been considered remote. With most plant viruses this may be true as with those sometimes consumed in infected raw vegetables including lettuce mosaic, celery mosaic, carrot mottle and, in brassicas, the cauliflower and turnip mosaic viruses. This is because, in infective plant tissue extracts, these viruses would be rapidly inactivated at 25 $^{\circ}\text{C}$ in solutions of pH 4.0–5.0. However, not all enteric pathogens need to be acid-stable—for example, *Vibrio cholerae* is not acid-stable—thus other agents present in a quantity of food could survive transit through the stomach. The more stable plant viruses such as the tomato and pepper strains of TMV may be sufficiently intrinsically stable to resist digestion, and experiments to test their possible 'alimentary resistance' will now be done. Similarly, the resistance of viroids¹⁰ for example, cucumber pale fruit and potato spindle tuber viroids, needs to be determined; although readily hydrolysed by pancreatic RNase *in vitro* in weak salt solutions (0.005 M phosphate buffer, pH 7.5), these are more resistant to the enzyme in solutions of higher molarity.

The results presented here suggest that man is a potential carrier of TBSV if he has consumed infected produce. The possibility exists that TBSV could enter rivers after sewage treatment of infective faeces but whether the virus is stable after this treatment is unknown, although its presence in tomato plants growing extraneously in primary settlement beds has been confirmed. Further dissemination might also occur by irrigation of susceptible crops with infective river water. Whether human factors (infective faeces) contribute to the incidence of TBSV in Moroccan⁴ and Tunisian (C. Cherif, personal communication) tomato crops merits further study. We shall now examine other plant viruses having no known vectors for their 'alimentary resistance', as man and other migratory animals may prove to be important carriers of these viruses also.

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Specific inhibition of the morphogenesis of *Trypanosoma cruzi* by a monoclonal antibody

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Trypanosoma cruzi, the causative agent of Chagas' disease, is transmitted primarily by blood-sucking reduviid bugs. The insects themselves are infected by ingesting trypomastigotes with the blood of infected mammals. On ingestion by the vector, the parasites convert to epimastigotes which multiply within the lumen of the crop and midgut. In the hindgut and rectum of the reduviid, the epimastigotes transform into non-dividing metacyclic trypomastigotes which can, when passed with the faeces, infect the vertebrate host¹. Lectins capable of binding *T. cruzi* have been isolated from different regions of the vector's intestinal tract^{2,3}. It has been proposed that the interaction of these lectins with the surface membrane of the parasite regulates the morphogenesis of *T. cruzi* in the gut of the vector^{2,3}. In the present study we describe the inhibition of epimastigote to trypomastigote differentiation *in vitro* by a monoclonal antibody specific for a 72,000 molecular weight cell-surface glycoprotein of *T. cruzi* and propose that this molecule may be a receptor controlling parasite differentiation in the vector.

The transformation of epimastigotes into trypomastigotes was studied in an *in vitro* assay which is described in detail elsewhere⁴. In this system, log phase epimastigotes (Tulahuen strain) cultured in liver infusion tryptose (LIT) medium are programmed for differentiation by inoculation into Millipore filter (0.45 µm) chambers which are then implanted subcutaneously in mice. When recovered 7 h after *in vivo* implantation these organisms are still in the epimastigote stage as judged by morphological criteria as well as their sensitivity to comple-

ment lysis and binding of specific lectins⁴. Nevertheless, on transfer to Eagle's minimal essential medium (EMEM) and incubation for 17 h at 37 °C in a 5% CO₂ atmosphere, 60–80% of the parasites transform into morphologically distinct trypomastigotes. This *in vitro* transformation is not observed when epimastigotes are cultured in the same conditions in the absence of prior *in vivo* programming⁴.

A series of six monoclonal antibodies reactive with the epimastigote surface and an epimastigote binding lectin, wheat germ agglutinin (WGA), were tested for their ability either to inhibit or promote epimastigote differentiation in the *in vitro* assay. The monoclonal antibodies were produced by fusing P3-NS1/1-AG-4-1 myeloma cells with spleen cells from BALB/c mice immunized with glutaraldehyde fixed epimastigotes as described previously⁵. With the exception of WIC 59.4, an IgM antibody, each of the monoclonal antibodies was purified from ascites by affinity chromatography on Protein A-Sepharose⁶. The purified antibodies (unpurified ascites in the case of WIC 59.4) were found to have indirect fluorescent antibody titres ranging between 10² and >10⁵ and to have parasite agglutination titres ranging between <10¹ and >10⁵ (Table 1). The epimastigote binding lectin, WGA, agglutinated the parasites at concentrations as low as 8 µg ml⁻¹.

When tested in the *in vitro* differentiation assay, only one of the reagents, an IgG2a monoclonal antibody WIC 29.26, significantly influenced the differentiation of epimastigotes into trypomastigotes. As shown in Fig. 1, the purified antibody significantly inhibited parasite transformation at dilutions as high as 10⁵ which is equivalent to an IgG to parasite ratio of 5 ng per 10⁶ organisms. No inhibitory activity was observed with the five other monoclonal antibodies at any of the concentrations tested. The effect of a representative negative antibody (WIC 29.19) with high anti-epimastigote binding activity is shown in Fig. 1. Similarly, no inhibition of transformation was observed with WGA even at concentrations as high as 250 µg ml⁻¹ (data not shown).

A trivial explanation of the inhibitory effect of WIC 29.26 on parasite transformation is that the antibody is trypanocidal or trypanostatic. Arguing against this hypothesis is the observation that incubation with WIC 29.26 causes no reduction in

Table 1 Monoclonal antibody reagents tested

Hybridoma	Ig class	Reciprocal IFA titre	Reciprocal agglutination titre
WIC 59.4	IgM	10 ⁵	10 ⁴
WIC 14.19	IgG1	10 ³	10 ²
WIC 29.19	IgG1	>10 ⁵	>10 ⁵
WIC 29.82	IgG3	10 ²	<10 ¹
WIC 53.1	IgG2a	10 ²	10 ¹
WIC 29.26	IgG2a	>10 ⁵	10 ³
	Fab	>10 ⁵	<10 ¹

Indirect fluorescent antibody (IFA) titres were determined by adding log dilutions of Protein A-purified monoclonal antibodies or papain-digested Fab fragments (starting concentration of approximately 5 mg ml⁻¹ to epimastigotes collected from LIT medium. The parasites were then incubated for 1 h at 37 °C, washed twice and incubated for an additional 30 min at room temperature with a 1/40 dilution of fluorescein-conjugated rabbit anti-mouse heavy and light chains (Cappel). After further washing, the parasites were examined in a fluorescence microscope. Direct agglutination of parasites was assayed by adding log dilutions of purified monoclonal antibody (or Fab fragments) to LIT derived epimastigotes (5 × 10⁶ per ml) in EMEM plus 1% bovine serum albumin in round-bottom microtitre trays. After 1 h at 37 °C, agglutination of the parasites was scored by examination with an inverted microscope.

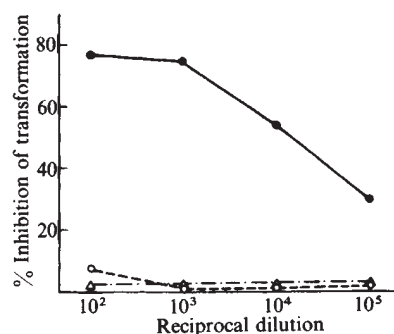


Fig. 1 Inhibition of epimastigote to trypomastigote transformation by monoclonal antibody WIC 29.26. Epimastigotes (Tulahuen strain) were collected from log phase cultures in LIT medium containing 10% fetal calf serum, washed in LIT, and inoculated into Millipore chambers which were then implanted subcutaneously into C57BL/6J mice. Seven hours later the parasites were recovered from the chambers, washed in EMEM and resuspended in EMEM to a final concentration of approximately 10⁷ epimastigotes per ml. Aliquots (45 µl) of the parasite suspension were then cultured together with 5 µl of monoclonal antibody or Fab preparation in EMEM for 17 h at 37 °C in a humidified atmosphere of 5% CO₂. The contents of duplicate microwells were then smeared, Giemsa stained and the percentage of mature trypomastigotes plus partially transformed trypomastigotes determined by morphological examination⁴. In the representative experiment shown, 76% of the parasites cultured in the absence of inhibitor differentiated into trypomastigotes. The effect of the following Protein A-Sepharose-purified antibody preparations (used at an initial concentration of 5 mg ml⁻¹) is shown: WIC 29.26 IgG2a (●), WIC 29.26 Fab (△), WIC 29.19 IgG1 (○).

epimastigote motility or in the number of total parasites recovered at the end of the 17-h assay period and that upon transfer to LIT medium after incubation in WIC 29.26 antibody parasites display subsequent growth kinetics indistinguishable from those exhibited by programmed epimastigotes or programmed epimastigotes pre-incubated with a control monoclonal antibody (WIC 29.19) (Fig. 2). Similarly, during the 17-h incubation with WIC 29.26 antibody, no inhibition of total protein synthesis (as measured by ^{75}Se -selenomethionine incorporation) is observed nor do major differences appear in the pattern of labelled proteins (as analysed by SDS-polyacrylamide gel electrophoresis) when parasites incubated in the presence or absence of WIC 29.26 antibody are compared (data not shown).

In order to investigate further the mechanism by which WIC 29.26 inhibits trypomastigote differentiation, monovalent Fab fragments were prepared from the purified antibody by papain digestion. Although the Fab preparation displayed the same indirect fluorescent antibody titre for epimastigotes as the bivalent WIC 29.26 (Table 1) it failed to inhibit transformation in the *in vitro* assay (Fig. 1).

The above findings demonstrate that monoclonal antibody WIC 29.26 potently and specifically inhibits the transformation of epimastigotes into trypomastigotes. The epimastigote surface antigen recognized by WIC 29.26 antibody has already been identified⁵. The antigenic determinant resides on the carbohydrate portion of a 72,000 molecular weight glycoprotein⁵ which is present on the surface of epimastigotes and metacyclic trypomastigotes but absent on bloodstream trypomastigotes and amastigotes (D.S., unpublished data). Although the exact role of the 72,000 molecular weight glycoprotein in parasite differentiation remains to be elucidated, the failure of WIC 29.26 monovalent Fab fragments to inhibit transformation (Fig. 1) suggests that cross-linking of the molecule is essential for the biological effect.

Our data predict the occurrence of a substance in the gut of the invertebrate host with a binding specificity and biological activity similar to the WIC 29.26 monoclonal antibody. Indeed, as mentioned earlier, lectins which react specifically with different morphological stages of *T. cruzi* have already been identified in different portions of the reduviid intestinal tract^{2,3}. If present in the lining of the crop and midgut of the vector, a lectin reactive with the carbohydrate moiety recognized by WIC 29.26 antibody, could inhibit transformation and thereby regulate the multiplication and accumulation of epimastigotes in this anatomical site.

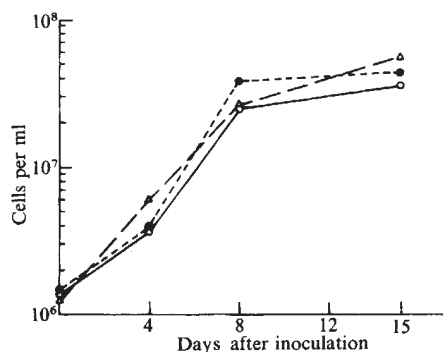


Fig. 2 Incubation with WIC 29.26 antibody has no effect on parasite viability or subsequent growth in culture. *In vivo* programmed epimastigotes incubated in EMEM for 17 h in the absence of inhibitors (○) or in the presence of a 1/1,000 dilution of either WIC 29.26 (△) or WIC 29.19 (●) monoclonal antibody were diluted 1:10 in LIT medium plus 10% fetal calf serum and the growth of the parasites assayed by haemocytometer counts. The results are the mean of assays on duplicate cultures. The absence of a significant lag in the growth of control, programmed cultures probably reflects rapid transformation of non-dividing trypomastigotes into epimastigotes during the first 4 days of culture.

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Alteration in the penicillin-binding profile of *Bacillus megaterium* during sporulation

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The development of convenient methodology to study penicillin-sensitive enzymes (PSEs) as penicillin-binding proteins (PBPs)^{1,2} has revealed their roles in bacterial cell shape determination, particularly in the Gram-negative rod *Escherichia coli*³⁻⁶. By analysing PBPs during the life cycle of *Bacillus megaterium* KM we report here the first data suggesting specific roles for these proteins in the morphogenesis of a Gram-positive rod. During the transition from the vegetative rod to the spherical/ellipsoidal-shaped dormant spore, novel PBPs are synthesized and existing ones are proteolytically modified. The differentiation of the dormant spore PBP profile back to that of the vegetative cell during germination, follows a defined sequence clearly correlated to specific changes in shape, culminating in a highly synchronous vegetative cell division.

The sporulating bacteria offer one of the most attractive prokaryotic systems to explore the cellular role of PBPs. In appropriate growth conditions, these bacteria can be induced to undergo a limited number of central septations in a synchronous manner (vegetative growth) followed by one final synchronous septation which differs from all previous septations by being asymmetrically positioned close to one cell pole⁷. Following this unequal partitioning of the cell which is unique to sporulating cells, the membranes comprising this asymmetrical septum continue to proliferate towards the nearest cell pole with the result that a separate spherical/ellipsoidal compartment (forespore) containing at least one complete genome is created within the parent cell (mother cell)⁸. By the nature of its formation, the forespore is enclosed by two membranes of opposite polarity⁹. During the next 12 h, in a series of well defined morphological and biochemical changes programmed by transcription of spore-specific genes, this forespore is converted into a mature spore¹⁰. The overall sporulation process is divisible into seven distinct stages (0-VI)^{11,12}. Stage II describes the asymmetric septation event and stage III denotes the first appearance of the discrete forespore. Of particular interest to the question of cell shape determination is the fact that within the cylindrical mother cell, a spherical/ellipsoidal layer of peptidoglycan (spore cortex) is synthesized between the two bounding membranes of the forespore during stages III and IV¹³⁻¹⁶. In addition to its altered shape, the chemistry of this cortex peptidoglycan is radically different from that of the parent cell^{14,17}. Thus, the sporulating cell not only provides an opportunity to investigate the switch from symmetrical to asymmetrical division and hence to probe polarity mechanisms, but also to study the chemical and enzymatic basis of a transition from cylindrical to spherical/ellipsoidal shape. Interestingly,

these transitions can also be investigated in reverse as dormant spores can be triggered to germinate and outgrow synchronously^{8,17,18}. Approximately 180 min after germination triggering, the peptidoglycan composition has reverted to that of a typical vegetative cell, the cells have regained their cylindrical shape and the population undergoes its first synchronous central septation^{16,18,19}.

We analysed PBPs in membranes isolated from vegetative cells (Fig. 1a), mother cells (Fig. 2) and forespores during sporulation (Fig. 1b-e), and from dormant (Fig. 1f) and germinated spores (Fig. 1g-h). Vegetative membranes of this sporogenic *B. megaterium* KM contained five PBPs identical both in their mobilities on SDS-polyacrylamide gel electro-

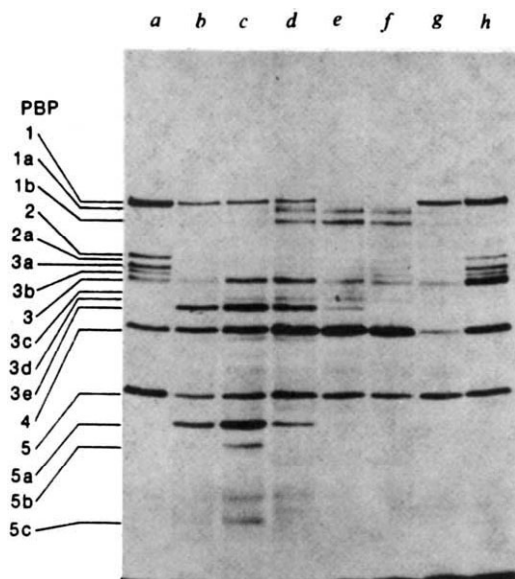


Fig. 1 Fluorogram of PBPs of membranes isolated from vegetative cells, forespores and dormant and germinated spores of *B. megaterium* KM. The conditions for growth and sporulation were as described previously^{27,28}. Intact cells (exponentially growing vegetative cells, isolated forespores, dormant and germinated spores) were disrupted by homogenization with glass beads in 0.05 M Tris-HCl pH 7.5 buffer, containing 2 mM phenylmethanesulphonylfluoride (PMSF)²⁹. Cell walls or integuments were sedimented (27,000 g for 3 min) and integument fractions²⁹ from stage V and VI forespores and dormant spores were washed four times by resuspension in 0.05 M Tris-HCl pH 7.5, 5 mM MgCl₂. The integument fractions isolated from developing and mature spores contained the outer forespore/spore membrane trapped between the proteinaceous spore coats and the cortex from stage V onwards³⁰⁻³². The supernatant fraction was centrifuged twice (27,000 g for 3 min) and the final supernatant was further centrifuged (105,000 g for 60 min) to sediment membranes, which were washed twice by repeated centrifugation and resuspended in 0.05 M Tris-HCl pH 7.5, 5 mM MgCl₂ buffer. In the preparation of the membranes from the sporulation stages, forespores were released after the lysozyme digestion step and the forespores and mother cell membranes isolated by differential centrifugation²⁸. The temperature of all preparations was rigorously maintained below 4°C. Freshly prepared dormant spores were germinated as described by Wilkinson *et al.*³³ in a modified sporulation medium (CCY medium) at a cell density of 0.5 mg dry weight ml⁻¹. The medium contained glutamine (50 mg l⁻¹), acid casein hydrolysate (2.5 g l⁻¹), enzymatic casein hydrolysate (2.5 g l⁻¹), yeast extract (1.0 g l⁻¹), glycerol (1.5 g l⁻¹) plus germinants (10 mM L-alanine and 5 mM inosine). Salts, at the normal concentrations of CCY medium²⁷, were added 45 min after the time of transfer of spores to the medium (*t*₄₅). Germination, outgrowth, elongation and the first vegetative cell division were monitored by phase contrast microscopy and membranes were prepared from cells at *t*₈₅ (elongation) and *t*₂₀₀ (cell division). Membranes, at 5–10 mg protein per ml, were incubated at 37°C for 10 min with ³H-benzylpenicillin (25 µg ml⁻¹; 31 Ci mmol⁻¹) in 0.05 M Tris-HCl pH 7.5, 5 mM MgCl₂ buffer. After reaction, samples were supplemented with a 1,000-fold excess of nonradioactive benzylpenicillin and an equal volume of SDS sample buffer (0.05 M Tris-HCl, pH 7.5, 2% (w/v) SDS, 0.05 M dithiothreitol, 4 mM PMSF, 2 mM EDTA, 20% (w/v) glycerol, 0.005% (w/v) bromophenol blue), heated at 100°C for 3 min, and subjected to 10% SDS-PAGE as described previously²⁹. Equal aliquots of protein, estimated by the method of Lowry *et al.*³⁴, were loaded on each track. PBPs were detected by fluorography³⁵, using pre-flashed Fuji X-ray film. a, Vegetative plasma membranes; b, inner (ifm) and outer (ofm) forespore membranes of stage III; c, stage IV, ifm and ofm; d, stage V, ifm; e, stage VI, ifm; f, dormant spore inner membrane; g, h, plasma membranes of germinated spores at *t*₈₅ and *t*₂₀₀, respectively.

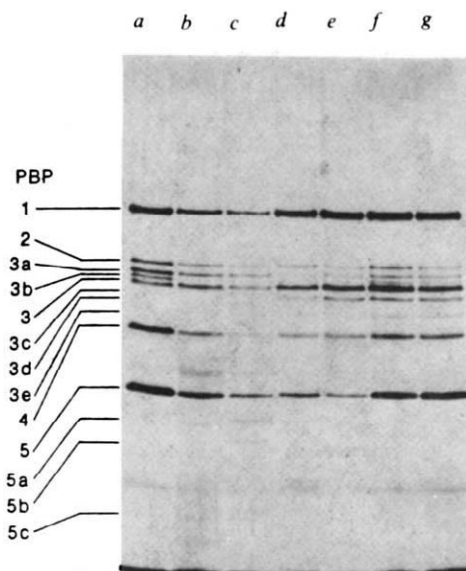


Fig. 2 Fluorogram of PBPs in the plasma membranes isolated from vegetative cells (a); sporulating cells at stage I (b) and stage II (c); and mother cells at stage III (d), stage IV (e), stage V (f) and stage VI (g). Mother cell plasma membranes, separated from the forespore membranes by differential centrifugation (see Fig. 1), and total cell membranes were prepared and assayed for PBPs as described in Fig. 1 legend.

phoresis (SDS-PAGE) and in their affinities for benzylpenicillin, to PBPs 1–5 of an asporogenic *B. megaterium* KM strain²⁰. In addition, four previously undetected PBPs were identified and designated PBPs 3a, 3b (Fig. 1a), 3c and 3d (Fig. 1d). The apparent molecular weights (MWs), affinities for benzylpenicillin and specific amounts of the PBPs in vegetative and forespore membranes of *B. megaterium* KM, are shown in Table 1.

During sporulation and, significantly, during cortex synthesis, dramatic changes in the number and level of PBPs were observed in the forespore membranes compared with the plasma membranes isolated from the corresponding mother cell (compare Fig. 1b–e and Fig. 2d–g). Novel PBPs were designated PBP 1a (doublet), 1b, 2a, 3e, 5a, 5b and 5c (Figs 1, 2). PBPs 5b [MW ~ 41,000 (41 K)] and 5c (MW ~ 39 K) were not characterized further. These changes in PBPs are almost certainly sporulation-specific and not a response to nutrient limitation, as an asporogenic *B. megaterium* KM strain²⁰ grown in sporulation medium for the same time as an equivalent stage IV culture, did not show any significant changes in PBPs (data not shown).

Peptide maps of PBPs 1a and 1b, obtained by partial proteolysis with staphylococcal V8 protease, were identical to that of PBP 1 (Fig. 3), suggesting a common structural identity. Similarly, on appropriately exposed fluorographs it is apparent that PBP 3c may be related to PBP 1, PBP 2a to 2 and PBP 3b to 3a (data not shown). In contrast, PBPs 3e and 5a have unique peptide profiles suggesting that these proteins may be both novel and sporulation-specific as opposed to proteolytic fragments derived from higher molecular weight PBPs. Furthermore, limited proteolysis of PBP 5a with α -chymotrypsin (Fig. 3c) and papain (Fig. 3d) produced peptides distinct from those of PBP 5 and PBPs 3e, 1, 3 and 4 (Fig. 3c, d).

In preliminary experiments (J.T. and D.E., in preparation), PBP 5a showed D-alanine carboxypeptidase activity with a pH optimum similar to the vegetative cell membrane carboxypeptidase, which is attributed to PBP 5 (ref. 20). *p*-Chloromercuribenzoate (PCMB) inhibits the binding of penicillin only to PBPs 5, 5a and 3e (data not shown). PBP 3e was thus clearly distinguished from higher molecular weight PBPs by virtue of this sensitivity to PCMB, its lower affinity for benzylpenicillin (Table 1) and a completely distinct peptide profile (Fig. 3). It is likely, therefore, that PBP 3e represents a unique PSE active in the synthesis of spore-specific

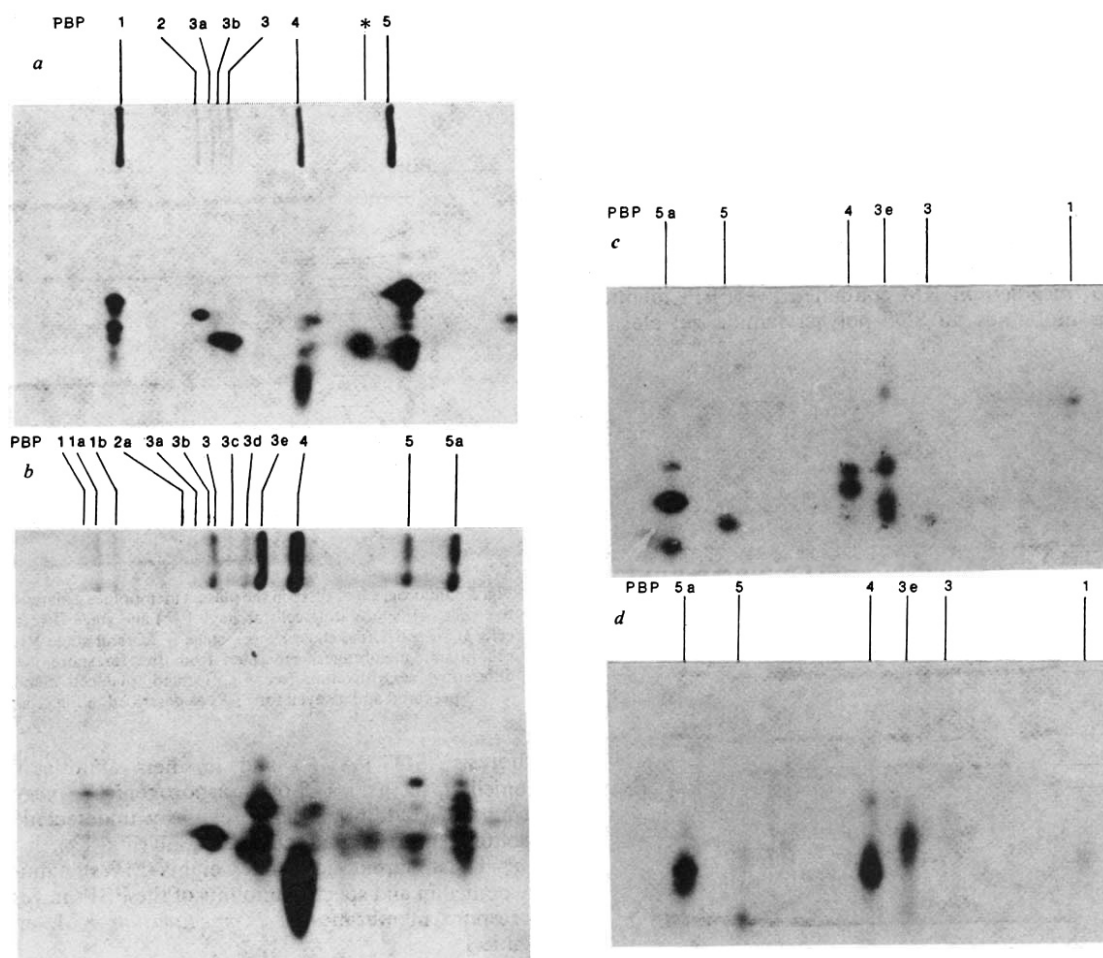


Fig. 3 Fluorograms of ^3H -benzylpenicillin-labelled peptides (obtained by partial proteolysis of PBPs with 100 μg of *Staphylococcus aureus* V8 protease) from vegetative plasma membranes (a) and stage VI forespore inner membranes (b). (c) and (d) show the peptide maps of PBPs 1, 3, 3e, 4, 5 and 5a obtained by partial proteolysis of stage IV forespore inner membranes with 125 μg α -chymotrypsin and 500 μg papain, respectively. The method used was based on that of Cleveland *et al.*³⁶, as modified by G. Stewart and D.J.E.³⁸. Membrane protein (75–100 μg) was reacted with ^3H -benzylpenicillin (50 $\mu\text{g ml}^{-1}$) and separated by 10% SDS-PAGE as described in Fig. 1 legend. An 18% acrylamide gel was prepared with a 5-cm 6% acrylamide stacking gel. A 2-cm space was left above the stacking gel to accommodate the one-dimensional track which was overlaid with 0.5 ml of stacking gel containing the protease. Electrophoresis was as described previously²⁹ except that the current was switched off for 30 min just before the bromophenol blue entered the 18% gel. The gels were prepared for fluorography with sodium salicylate, as described by Chamberlain³⁷. Duplicate tracks from the first dimension gel were fluorographed and shown in the figure as markers for each PBP in the peptide map. * The labelled peptide in this position is a result of nonspecific penicillin binding to a major membrane protein.

peptidoglycan. The second sporulation-specific protein, PBP 5a, may regulate the degree of cross-linkage (10-fold lower in spore peptidoglycan than in vegetative peptidoglycan^{14,21}) and/or provide a suitable tetrapeptide substrate for the transpeptidase synthesizing spherical/ellipsoidal peptidoglycan at stage IV. PBP 5a may thus be analogous to PBP 5 in rod-shaped *E. coli* which, when overproduced, results in spherical growth²².

In addition to the characteristic differences observed at stage V between the inner forespore membrane (ifm) (Fig. 1d) and the mother cell membrane (Fig. 1f), the outer forespore membrane (ofm) at this time is devoid of PBPs 1, 1a, 1b, 2, 2a, and 3a–3d and contains only 30% of the total forespore penicillin-binding capacity (data not shown). This suggests that the forespore compartment is directing the synthesis of novel PBPs into the inner forespore membrane and may indicate a role for the forespore cytoplasm and inner membrane in cortex assembly. A similar role was previously suggested by Frehel and Ryter¹⁵ on the basis of their autoradiographic studies.

The re-establishment of the vegetative rod shape by a combination of swelling (t_0 – t_{45}) and elongation (t_{45} onwards) during germination, was accompanied by a return to a PBP profile resembling that of the vegetative cell. This was achieved by a specific decrease in PBP 4 from t_{60} and the reappearance of PBP 1 from t_{45} as demonstrated by the PBP profile of cells at t_{85} (Fig. 1g). These data suggest that PBP 4 functions specifically

in the synthesis of spherical peptidoglycan during t_0 – t_{60} while PBP 1 may be concerned with polymer synthesis during elongation (a fuller characterization of germination-associated changes in PBPs will be presented elsewhere). The latter proposal is consistent with that of Reynolds *et al.*²³, who have suggested that PBP 1 is the major transpeptidase of *B. megaterium* KM. Hence, the substantial reduction in PBP 1 and the appearance of its proteolytic fragments, PBPs 1a and 1b, during sporulation may indicate a control mechanism that suppresses vegetative cell elongation in the stage III forespore as a prerequisite for the synthesis of spherical/ellipsoidal peptidoglycan. The role specified here for PBP 4 in the synthesis of spherical-shaped peptidoglycan is consistent with the results of Taku *et al.*²⁴. They purified a PBP (MW ~ 61 K) from *B. megaterium* 899 having transglycosylase activity *in vitro* but with no demonstrable transpeptidase activity, which probably corresponds to PBP 4 of *B. megaterium* KM. Because PBPs 1a, 1b and 3 of *E. coli* are probably the transpeptidase/transglycosylase enzymes responsible for lateral wall and septum peptidoglycan synthesis respectively^{3–6}, Taku *et al.*²⁴ have suggested analogous enzyme activities and roles in cell growth of *B. megaterium* for PBPs 1 and 4.

The present paper provides the first experimental support for the hypothesis that PBPs/PSEs have central and distinct roles in the morphogenesis of Gram-positive rod-shaped bac-

Table 1 PBPs of the membranes from vegetative and sporulating cells and dormant and germinated spores of *B. megaterium* KM

Specific levels of PBPs (no. of PBP molecules per 10 ⁻¹⁵ g of protein).												
PBP	Apparent MW (K)	Benzylpenicillin affinity (μg ml ⁻¹)	Exp	Membranes						DS (im)	GS t ₈₅	GS t ₂₀₀
				I	II	III (ofm + ifm)	IV (ofm + ifm)	V (ifm)	VI (ifm)			
1	115	0.04	5.0	1.3	0.8	0.8	0.1	0.1	0.1	+	1.0	3.6
1a	112	0.04	—	—	—	+	+	0.4	0.7	0.8	0.1	+
1b	107	0.04	—	—	—	+	+	0.4	1.1	1.2	0.1	+
2	92.5	0.10	1.0	0.7	0.2	+	+	+	—	—	0.2	0.7
3a	87.5	0.1	1.7	0.7	0.4	+	+	+	0.1	0.2	0.1	1.1
3b	85.5	0.1	0.8	+	0.1	+	+	+	0.1	0.2	0.1	1.1
3	84	0.3	1.0	1.3	0.7	0.3	2.5	1.0	0.7	0.7	0.4	3.5
3c	79.5	0.04	+	+	+	0.1	0.1	0.1	0.2	0.2	0.1	+
3d	76	0.01	+	+	+	0.1	0.1	0.1	0.4	0.8	0.1	+
3e	73.5	0.5	—	—	0.2	3.0	6.6	3.5	0.4	—	—	—
4	67	0.3	2.9	1.0	0.3	2.1	4.1	6.4	9.0	11.3	1.2	5.3
5	50	2.0	4.2	3.0	1.2	1.7	1.7	1.3	2.0	1.0	2.7	5.1
5a	44.5	2.0	—	—	0.4	3.8	10.1	2.0	0.1	—	—	—

Membranes were prepared from exponentially growing vegetative cells (Exp), stage I and II sporulating cells, stage III, IV, V and VI forespores, dormant (DS) and germinated spores (GS, t₈₅ and GS, t₂₀₀) as described in Fig. 1 legend. Stage III and IV forespore membranes were a mixture of the ofm and ifm; stage V and VI forespore membranes and dormant spore membranes were, respectively, the ifm and spore inner membrane (im) only. The values of the affinities for benzylpenicillin of PBPs 1, 2, 3, 4 and 5 were taken from Chase *et al.*²⁰. The affinities of the other PBPs for benzylpenicillin were estimated as described previously²⁰ (PBP 2a (MW ~90 K) has an affinity of 0.1 μg ml⁻¹, data not given). Molecular weights were estimated by the relation of log MW to the R_f of ¹⁴C-methylated protein standards (Amersham). PBPs were quantified by estimation of the amount of radioactivity associated with the appropriate gel slice after location by fluorography (see Fig. 1 legend). Gel slices were prepared for liquid scintillation counting by the method of Tishler and Epstein²⁶. Slices were solubilized by incubation at 50 °C for 24 h with 0.3 ml (30%/100 vols) of hydrogen peroxide. Determination of radioactivity was as described previously²⁷. The number of PBP molecules per 10⁻¹⁵ g protein was calculated from the specific activity of the label.—Denotes that the PBP was not detectable after prolonged exposure of the fluorogram and + denotes that the counts associated with the PBP were insufficient to permit quantitation.

teria in a manner analogous to those of *E. coli*^{3-6,22,25}. It also establishes PBPs 3e and 5a as unique sporulation-specific membrane-bound enzymes. The differentiation of PBPs during *B. megaterium* KM development should provide an ideal system to study differential gene expression in two separate intracellular compartments of a prokaryote and the mechanisms by which these proteins determine bacterial cell shape.

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Elucidation by FAB-MS of the structure of a new cardioactive peptide from *Aplysia*

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The nervous system and gut of *Aplysia* and other gastropod molluscs contain several classes of cardioactive peptides¹. In addition to their excitatory action on the heart, these peptides stimulate contractile activity in the gut¹, and modulate neurally induced contractions of somatic muscles². This report describes the purification and sequence determination by a combination of microprotein chemistry and fast atom bombardment mass spectrometry FAB-MS^{3,4} of one of these peptides. The sequenced peptide is a member of the small class of cardioactive peptides and was termed SCP_B. A peptide with identical properties to SCP_B has been found in several identified central neurones which innervate the gut⁵. Here we report the structure of SCP_B as a C-terminally blocked peptide

H-Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH₂

Native and synthetic SCP_B have identical actions on molluscan muscle preparations with thresholds at around 10⁻¹⁰ M.

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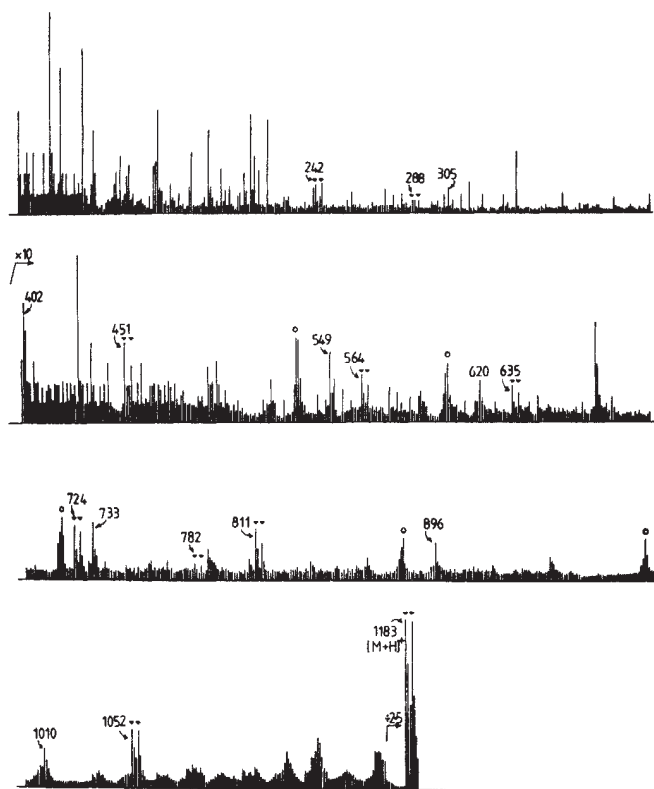
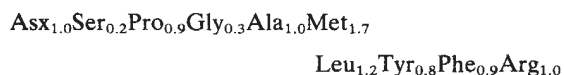


Fig. 1 Fast atom bombardment mass spectrum of the *N*-(1:1 acetyl: d_3 -acetyl) derivative of SCP_B (1 μ g). Ions arising from the *N*-terminus appear as 1:1 doublets, three mass units apart. The doublets and C-terminal singlets have been marked for clarity.

The central nervous systems from 2,500 *Aplysia brasiliana* were used as starting material. The homogenization, desalting and gel filtration steps were as previously described⁶. After each step, the active fractions were located by their inotropic action on an isolated snail heart assay⁷. The active fractions were applied to a cation-exchange column of SP-Sephadex (Pharmacia) at pH 6.0, and a linear gradient applied from 10 mM NH_4 acetate (pH 6.0) to 100 mM NH_4 acetate, 100 mM NH_4OH (pH 9.6). The cardioactive material eluted as a single peak which was further purified by HPLC. The sample was taken up in 0.1% trifluoroacetic acid (TFA) and applied to a Bondapak C18 column (Waters). A reverse phase gradient was developed from the sample buffer to 0.07% TFA in acetonitrile. Two active peaks termed SCP_A and SCP_B were obtained (evidence from FAB-MS analyses suggests that the peak termed SCP_A contains the oxidized form of SCP_B which may account for its biological activity). Quantitative amino acid analysis suggested that SCP_B was a single peptide species with the following amino acid composition normalizing on Arg = 1:



The recovery from the final purification (1,000 animals) was 15–20%, with a yield of 10 nmol. Thus the original content of SCP_B in each nervous system (animal weight 400 g) was approximately 60 pmol.

After biological experiments and amino acid analyses 6 nmol were available for the mass spectrometric study. Each experiment was therefore restricted to 1.5 nmol (approximately 1 μ g of SCP_B per analysis). The first sample was examined directly by FAB-MS as the free peptide, and gave a clear signal at 1,141, indicating a molecular weight for SCP_B of 1,140. This

was in approximate agreement with that anticipated from amino acid analysis. Sequence ions in the spectrum were weak and the complexity did not allow an interpretation. A further 1 μ g of SCP_B was treated with acetic anhydride: d_6 acetic anhydride 1:1 v/v in methanol for 1 min at room temperature⁸, followed by FAB-MS examination. The spectrum produced is shown in Fig. 1.

The appearance of a 1:1 signal (containing an acetyl group) at m/e 1,183 suggested that the *N*-terminus was not blocked, although abnormal acetylation of a very reactive guanidino group could not be eliminated at this stage. (Spinning-cup sequencer studies on the related SCP_A proved negative suggesting a possible blocked *N*-terminus). Importantly, the presence of weak 1:1 doublets throughout the spectrum now allowed a partial sequence of SCP_B to be built up by looking for meaningful mass differences based upon empirical FAB fragmentation mechanisms⁴. For example, the doublets at m/e 242, 288, 451, 564, 635, 724, 782 and 811 were spotted and have been marked on Fig. 1 for clarity. Not all of these led to readily interpretable mass differences but the signals at m/e 288, 451, 564, 635 and 782 gave a tentative partial sequence of



(where X and Y denote unknown portions of the molecule). Note that the signals at m/e 724 and 811 are not assigned and in fact were never used in the sequence assignment. They were assumed to be quasimolecular ions of contaminating peptides and this was later confirmed by their absence from the spectrum of the synthetic peptide.

At this stage, 1 μ g of SCP_B was digested with trypsin pH 8.3 (1:50 enzyme to substrate, at 37°C for 1 h) and the digest examined directly by FAB-MS. This experiment was crucial for the interpretation of the structure (Fig. 2). A new quasimolecular ion is seen at m/e 1,011, concomitant with the disappearance of the original signal at m/e 1,141. The new peptide giving rise to the 1,011 signal would be expected to contain a C-terminal arginine residue and its high mass placed that arginine towards the C-terminal end of the molecule. However the mass difference of 130 AMU lost on digestion does not correspond to any single amino acid or combination of amino acids. However, the mass difference is just one atomic mass number away from the mass of methionine. If we then postulate that SCP_B has a blocked amidated C-terminus, the tryptic digestion process would liberate a free carboxyl group from a previously amidated extended sequence, thus accounting for the one atomic mass unit and we therefore deduced that the SCP_B peptide contained a C-terminal-Arg-Met- NH_2 unit.

Because the 1:1 labelled signals (m/e 288 etc.), which appear to be derived from partial sequence -Tyr-Leu-Ala-Phe-, were not C-terminal ions extending N-terminally from the Arg-Met-amide sequence, it then followed that the label could not be on the arginine but was on the amino terminus and thus SCP_B was not N-terminally blocked.

Using this information we looked for C-terminal sequence ions in Fig. 1 and noted signals at m/e 549, 620, 733, 896 and

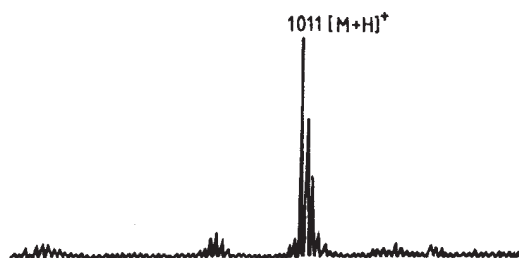


Fig. 2 Molecular ion region of the fast atom bombardment mass spectrum of the tryptic digest of SCP_B (1 μ g), showing the presence of a quasimolecular ion $(M+H)^+$ at m/z 1,011.

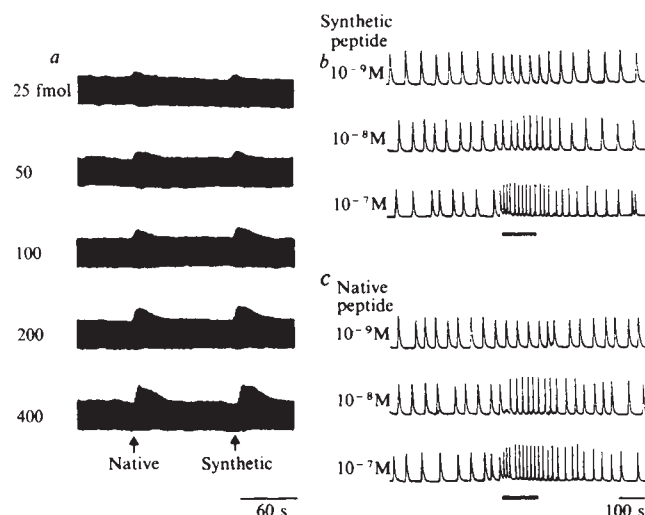


Fig. 3 A comparison of the biological activities of native and synthetic SCPBs on two preparations. *a*, Pulse applications of 25–400 fmol of the peptides to an isolated snail heart. Native and synthetic peptides caused similar increases of the amplitude of heart beat contractions at each concentration. *b*, *c*, Superfusion of an isolated snail oesophagus with 10^{-9} – 10^{-7} M synthetic SCPB (*b*), and native SCPB (*c*). The contractile activity of the oesophagus represents rearward directed peristalses which increase in frequency when either the synthetic or native SCPB is present in the bath (at horizontal bars).

1,010. These signals are all accompanied by satellites 15 AMU lower corresponding to the alkyl rather than the amine fragment with the charge on the C-terminus. This pattern often facilitates the identification of C-terminal sequence ions. By interpreting mass differences the sequence -Asn-Tyr-Leu-Ala has been generated.

The unknown unit 'X' on the N-terminal side of the tyrosine residue had a mass of 288 AMU, so by using C-terminal signals we have deduced that one component of this signal is asparagine. It now follows by mass difference that the N-terminal amino acid of SCPB is methionine, thus giving a structure H-Met-Asn-Tyr-Leu-Ala-

Combining these data with the N-terminal assignment and trypsin experiment now extends the sequence to



Using similar logic to that above, on any of the C-terminal signals, for example m/e 549, and subtracting the masses of Phe, Arg and Met amide, we conclude that the bracketed gap in the sequence above has a mass difference of 97 AMU which corresponds to proline. This assignment is confirmed by the observation of a signal at m/e 402 corresponding to the C-terminal signal Pro-Arg-Met-amide. Note that there is no satellite 15 AMU away, as would be expected since proline has a tertiary nitrogen, and cannot fragment to give an alkyl unit.

A combination of all the above analyses gives the structure of SCPB as the C-terminally blocked nonapeptide



The peptide was synthesised via *t*-butyloxycarbonyl (Boc) ester intermediates⁹ using *N,N*-dicyclohexylcarbodiimide-1-hydroxybenzotriazole coupling¹⁰. The amide group was introduced at the Boc-Pro-Arg-Met methyl ester stage by treatment with methanolic ammonia. The protected nonapeptide was purified by countercurrent distribution and deprotected in 90% TFA. Reverse-phase HPLC showed that the synthetic peptide was 95–97% pure, and that it precisely co-eluted with the native peptide.

The biological activities of the native and synthetic peptide were compared on two types of preparation; isolated heart and

oesophagus from snails. The isolated snail heart is the most quantitative of the available assays for SCPB, but responds in similar fashion to the application of other substances such as other neuropeptides and serotonin⁵. In contrast, the isolated snail oesophagus is less quantitative but responds in a qualitatively different fashion to SCPB, other neuropeptides and serotonin¹. Native and synthetic peptides were quantified by amino acid analysis and applied in known amounts to the heart (Fig. 3*a*) and oesophagus (Fig. 3*b*). These results demonstrate that the active and synthetic peptides are quantitatively and qualitatively identical. We have also carried out some preliminary experiments which indicate that synthetic SCPB also excites the isolated *Aplysia* heart (threshold 10^{-11} M). Together with the FAB-MS analysis of synthetic SCPB these data confirm that the presented sequence is correct.

It is interesting to note that (1) our sequence terminates with several hydrophobic residues, (2) the penultimate residue is arginine, and (3) the C-terminus is amidated. This is strikingly similar to another molluscan cardioactive peptide, Phe-Met-Arg-Phe-amide¹¹. Our work indicates that a new peptide class may exist, the key unit for biological activity being

Phe-A-Arg-B-amide

(where A and B are also hydrophobic amino acids), and probes designed with such a sequence may be valuable in the search for related peptides. Recent reports have suggested that there may be considerable conservation of neuropeptides between invertebrates and mammals, and there are a number of interesting cases where the original elucidation of a peptide class was carried out in an invertebrate. Examples of this include eladoin (a tachykinin) from octopus¹², FRMF-amide from clam¹³ and head-activating peptide from *Hydra*¹⁴. It will therefore be important to ascertain if peptides related to SCPB are present in other animals including mammals.

The precise physiological role of SCPB has not been determined in any gastropod mollusc, but it appears likely that it is involved in the control of gut activity^{1,5}. In addition its marked potency in exciting many different muscle preparations suggests that it may be involved in a more general arousal in these animals.

Finally, we note that mass spectrometry, whilst previously essential in the elucidation of such structures as the enkephalins¹⁵, leukotriene D¹⁶ and adipokinetic hormone¹⁷, has now improved in sensitivity by at least an order of magnitude. Although FAB-MS rarely gives complete sequence information at the 1 nanomole level, this paper demonstrates how a complete unknown sequence can be generated by judicious use of enzyme digests and micro-scale derivatization.

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Lectin binding reveals a synapse-specific carbohydrate in skeletal muscle

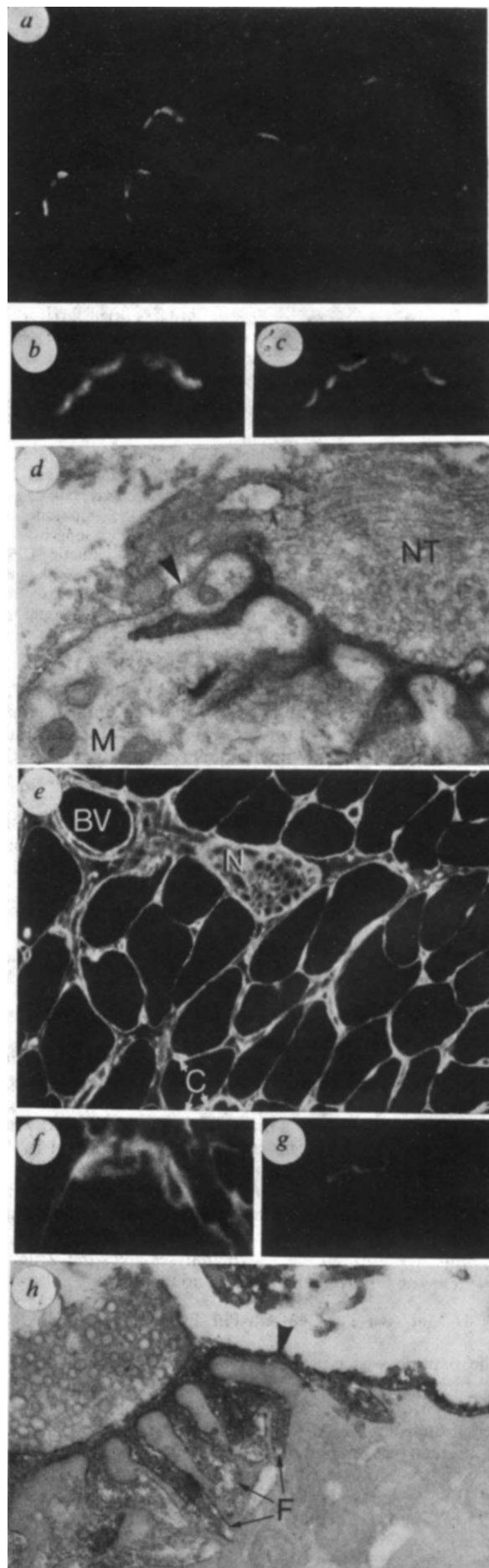
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Molecules concentrated at synapses are of interest because they may have roles in synaptic development or function. Several 'synapse-specific' proteins have been identified at the skeletal neuromuscular junction; acetylcholine (ACh) receptors¹, acetylcholinesterase², some components of the basal lamina³ and a number of cytoskeletal elements^{4,5}, are all present at far higher concentrations at synaptic sites than elsewhere on the muscle fibre surface. As carbohydrates are thought to be involved in neuronal recognition, adhesion and synaptogenesis⁶⁻¹², we have asked whether any saccharide moieties are similarly concentrated at synaptic sites on muscle fibres. Lectins are proteins that bind to carbohydrates; each has its own sugar specificity^{13,14}. Fluorescent and other derivatives of lectins have been used as histochemical stains to study the nature and distribution of carbohydrates on many cell types¹³⁻¹⁶ including neurones^{6,7,17-19} and muscle fibres^{16,20}. We report here that the lectin from the legume *Dolichos biflorus*, DBA, selectively stains neuromuscular junctions in several vertebrate species. This result demonstrates the existence of a synapse-specific carbohydrate.

Cryostat sections of unfixed skeletal muscle were incubated with fluorescein-conjugated lectins and viewed by fluorescence optics. Fluorescein-DBA stained only a few discrete spots on the surface of rat muscle fibres (Fig. 1a). Double staining with rhodamine- α -bungarotoxin, which binds to ACh receptors in the postsynaptic membrane^{1,3-5}, showed that the DBA-stained patches were synaptic sites (Fig. 1b,c); no other structures identifiable in cross-sections were stained by this lectin. Fluorescein-DBA also stained neuromuscular junctions specifically and intensely in cryostat sections of rabbit, hamster and chick muscle; frog (*Rana pipiens*) neuromuscular junctions were faintly, but selectively stained. Thus, DBA recognizes molecules that are highly concentrated or especially accessible at the neuromuscular junction.

Fig. 1 a-c, Staining of rat neuromuscular junctions by fluorescein-DBA. Cross-sections of frozen, unfixed rat diaphragm were incubated for 30 min with a mixture of fluorescein-DBA (E-Y Laboratories) and rhodamine- α -bungarotoxin, then photographed with appropriate filters to show fluorescein (a,b) or rhodamine (c). Rhodamine-bungarotoxin binds to ACh receptors in the postsynaptic membrane and thus marks synaptic sites³⁻⁵. b and c show the same field; the coincidence of fluorescein and rhodamine demonstrates that DBA stains synaptic sites. Similar results were obtained with fluorescein-DBA from Polysciences, and with biotin-DBA plus fluorescein-avidin from Vector Laboratories. d, Electron micrograph of a neuromuscular junction from a rat intercostal muscle incubated with peroxidase-DBA, then washed, fixed and stained for peroxidase (see ref. 27 for details). Reaction product is present in the synaptic cleft between the nerve terminal (NT) and muscle fibre (M), and in junctional folds (F; see h), but not on extrasynaptic portions of the muscle fibre surface (arrowhead). No staining was detectable when muscles were incubated without lectin. e-h, Sections prepared as in a-d, but stained with *Ricinus communis* I lectin. This lectin stains both synaptic and extrasynaptic portions of the muscle fibre surface, as well as surfaces of blood vessels (BV), capillaries (C), and axons and perineural sheaths of intramuscular nerve trunks (N). Scale bar, 50 μ m in a and e; 10 μ m in b, c, f and g; 0.5 μ m in d; and 0.4 μ m in h.



DBA is a lectin that recognizes *N*-acetylgalactosaminyl (GalNAc) termini of oligosaccharide chains^{13,21}. Three results provide evidence that the staining of rat neuromuscular junctions by fluorescein-DBA represents binding of the lectin to a synapse-specific carbohydrate that contains or resembles Gal-NAc. First, staining was abolished by addition of 20 mM Gal-NAc to the incubation medium (Fig. 2a) but persisted in the presence of 100 mM, D-glucose, D-galactose, *N*-acetylglucosamine or α -methylmannoside. Thus DBA binds to synapses via its GalNAc-binding site. Second, two other lectins that recognize GalNAc residues, *Glycine max* (soybean) lectin and *Vicia villosa* lectin, stained synaptic sites intensely. These two lectins also stained the extrasynaptic muscle fibre surface faintly, possibly because they are less discriminating than DBA in their preference for GalNAc over other sugars^{21,22}. Finally, lectins that recognize three other sugars do not distinguish synaptic from extrasynaptic regions; concanavalin A, wheat-germ agglutinin, and *Ricinus communis* I lectin (staining blocked by α -methylmannoside, *N*-acetylglucosamine and D-galactose, respectively) all stained both synaptic and extrasynaptic portions of the muscle fibre surface as well as the surfaces of blood vessels, capillaries, myelinated axons and intramuscular nerve sheaths (Fig. 1e-h). This result shows that specific staining of neuromuscular junctions by DBA is not due to an overall disproportion of carbohydrate at synaptic sites.

Further histochemical studies of rat muscle elucidated the localization of the synaptic component(s) to which DBA binds. Electron microscopy of muscle stained with peroxidase-DBA revealed reaction product in the synaptic cleft and in junctional folds but not elsewhere on the muscle fibre surface (Fig. 1d), confirming that this lectin binds selectively to synaptic sites. As muscles were incubated with peroxidase-DBA while alive, then washed, fixed and stained³, at least some DBA receptors must be external to the protein-impermeable plasma membrane. Staining persisted without any apparent diminution in denervated muscle (Fig. 2b), demonstrating that DBA receptors are not confined to the nerve terminal, but are present on the muscle fibre surface. DBA also stained synaptic sites after muscle was subjected to either of two treatments that remove cytoplasm and plasma membrane but not the basal lamina, that is; damage-induced degeneration and phagocytosis of muscle fibres (Fig. 2c) or salt and detergent extraction of sliced muscle³. This shows that some DBA receptors are associated with the basal lamina of the synaptic cleft; it does not, however, exclude the possibility that DBA also binds to some components of the plasma membrane. (Some receptors for concanavalin A, wheat-germ agglutinin and *Ricinus communis* I lectin also survived muscle damage and thus are associated with basement membrane in muscle, as they are in kidney²³.)

The identity and function of the DBA receptor(s) we have stained are unknown. GalNAc-binding lectins recognize glycoproteins, glycolipids and glycosaminoglycans^{13,21,24}, any or all of which might be concentrated at synaptic sites. Among many possible roles for the DBA-binding carbohydrates are targeting of molecules to synaptic sites¹², adhesion of nerve and muscle⁶, and recognition of synaptic sites by nerve terminals²⁵. An intriguing idea is that there is an endogenous lectin^{8,24} at the synapse which, in concert with the DBA receptor, plays some part in the formation or function of the neuromuscular junction.

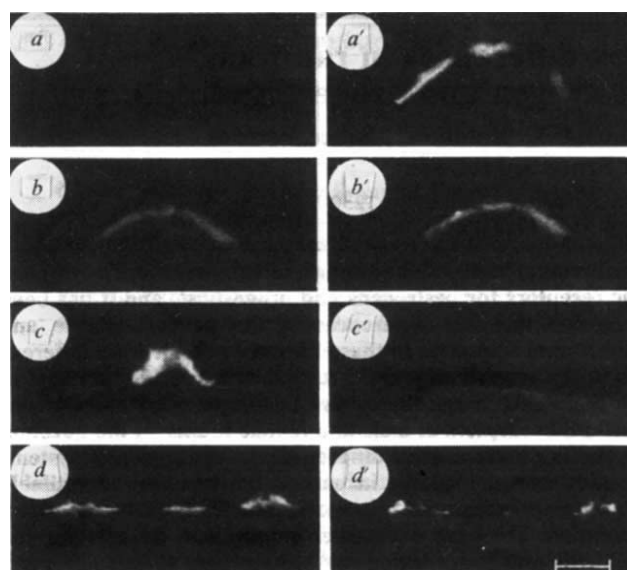


Fig. 2 Characteristics of DBA receptors in muscle. Muscles were doubly-labelled with fluorescein-DBA (or biotin-DBA followed by fluorescein-avidin) and rhodamine-bungarotoxin, as described in Fig. 1 legend. Each pair of micrographs shows the same field photographed with fluorescein- (a-d) and rhodamine- (a'-d') selective filters. a, a', Cryostat section of normal rat diaphragm stained in the presence of 20 mM GalNAc. The hapten sugar has blocked staining of the synaptic site by DBA; compare with Fig. 1b, b'. b, b', Cryostat section of rat diaphragm denervated 2 weeks earlier. Nerve terminals have degenerated and been removed, but DBA still stains synaptic sites. c, c', Cryostat section of rat intercostal muscle that had been damaged³ and denervated 3 days earlier. Nerve and muscle membranes have degenerated, and bungarotoxin-binding sites have disappeared, but basal lamina sheaths of muscle fibres survive (see refs 3, 27 for details); DBA stains synaptic sites on the sheaths. In other experiments, such DBA-stained patches were positively identified as synaptic sites by double staining with an antibody to a synapse-specific component of muscle fibre basal lamina⁵. d, d', Myotube from a chick nerve-muscle co-culture prepared as described by Fischbach²⁸ (provided by Drs L. Role and G. Fischbach). DBA binds to ACh receptor-rich patches on the myotube surface. Scale bar, 10 μ m.

These possibilities are best tested *in vitro*. In initial studies, we have found that fluorescein-DBA binds selectively (although not exclusively) to ACh receptor-rich regions of the myotube surface in chick nerve-muscle co-cultures (Fig. 2d); some of these patches are synaptic sites²⁶.

Thus, we have shown that the plant lectin DBA binds specifically to synaptic sites on the surface of skeletal muscle fibres; to our knowledge, this is the first demonstration of a carbohydrate moiety specifically localized at a synapse in any tissue. DBA should be a useful tool with which to study the identity, metabolism and function of this carbohydrate. In addition, DBA can serve as a simple light and electron microscopic stain for neuromuscular junctions.

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Sex differences in rat brain oestrogen and progestin receptors

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The brains of both male and female rats possess specific cytoplasmic receptors for oestrogens and progestins¹, and it has been suggested that sex differences in the neuroendocrine and behavioural responses to these hormones result from differences in the number of steroid receptors in specific locations in the brain². Although there have been numerous comparisons of steroid receptors in male and female brains³⁻⁹, the question of whether there are sex differences in these receptor systems remains unresolved, due in part to the inability of available biochemical techniques to achieve sufficiently high anatomical resolution. We have overcome this problem by applying the Palkovits punch-out technique¹⁰ to measure steroid receptors in hypothalamic nuclei known to be involved in behavioural and neuroendocrine control. We report here that castrated male rats have fewer oestrogen and progestin receptors in certain hypothalamic nuclei than females. This suggests that the relative insensitivity of male rats to feminine actions of gonadal steroids might be due in part to a lack of receptors for these steroids in highly localized brain regions.

Hypothalamic nuclei were removed from frozen, 300- μ m thick brain sections, using hollow stainless steel cannulas, 500 or 1,000 μ m in diameter. We pooled tissue from three ovariectomized or castrated rats (CD-1 strain, Charles River Biological Laboratories; 150–180 g) for an assay. For measurement of progestin receptors, all rats received subcutaneous (s.c.) injections of 10 μ g oestradiol benzoate (OB) once a day for 3 days before killing; this treatment induces hypothalamic progestin receptors to high levels^{11,12}. Rats were perfused through the heart with 10% ice-cold dimethyl sulphoxide to protect steroid receptors against loss from freezing¹³. We removed from the brain sections portions of the bed nucleus of the stria terminalis, the medial preoptic nucleus, the periventricular region of the preoptic area and of the anterior hypothalamic nucleus, the ventromedial nucleus and the arcuate nucleus. These six nuclei and their subregions were examined because they have the highest concentrations of gonadal steroid receptors in the brain^{14,15}, and have been extensively implicated in the behavioural and neuroendocrine actions of steroids¹⁶. The number of oestrogen and progestin receptors was measured in cytoplasmic fractions of the nuclei by the specific binding of ³H-OE₂ (115 Ci mmol⁻¹, NEN) and ³H-R5020, a potent synthetic progestin (87 Ci mmol⁻¹, NEN). Cytosol protein (8–50 μ g) was incubated with 1 nM ³H-OE₂ or 0.4 nM ³H-R5020 at 4°C for 4 h. For each nucleus, the amount of protein in the incubate was the same for males and females (Table 1). Non-specific binding was measured by co-incubation with 1 μ M moxestrol or 0.1 μ M R5020, respectively. The ³H-steroid bound to receptors was separated from free ³H-steroid by gel filtration on Sephadex LH-20 columns¹¹⁻¹⁵. The amount of protein in the incubate was determined by the method of Bradford¹⁷. The levels of oestrogen and progestin receptors in brain samples were expressed as steroid specifically bound per mg protein.

In preliminary studies, we observed a qualitatively similar distribution of gonadal steroid receptors in male and female brains. The highest levels of both oestrogen and progestin receptors in either sex were found in the periventricular region of the preoptic area, with substantial levels of binding also observed in the arcuate, medial preoptic and ventromedial hypothalamic nuclei, and in the periventricular region of the

anterior hypothalamic nucleus. The bed nucleus of the stria terminalis showed high oestrogen binding, but low levels of progestin receptors. We observed moderate amounts of gonadal steroid receptors in the anterior hypothalamic nucleus, the dorsomedial nucleus of the hypothalamus, and the medial nucleus of the amygdala. Both males and females had low levels of receptors in the lateral preoptic nucleus, the nuclei of the septum and the diagonal band of Broca. We also observed similar, low levels of uninduced progestin receptors in males and females.

Although the general receptor distribution was the same for males and females, there were substantial quantitative sex differences in the receptor content of specific nuclei (Fig. 1). The medial preoptic nucleus, which may be the target region for oestrogen-induced control of gonadotropin release¹⁶, had less than half the level of oestrogen receptors in males as in females (7×10^{-15} as opposed to 17×10^{-15} mol (moles) per mg protein). There was a large sex difference in the progestin receptor content of the ventromedial nucleus, with oestrogen-treated male rats having less than 30% of the receptor levels of oestrogen-treated females (8 as opposed to 25×10^{-15} mol

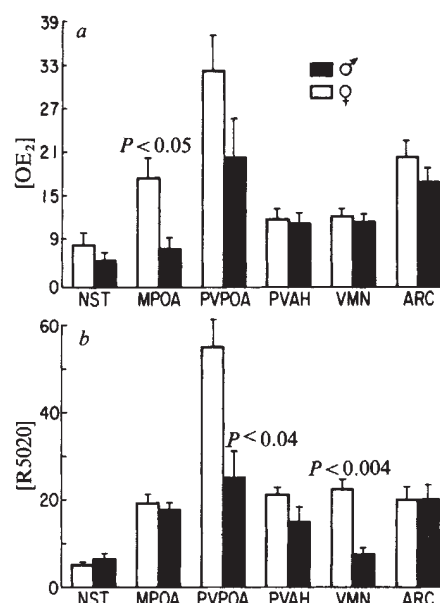


Fig. 1 Oestrogen and progestin receptor levels in hypothalamic nuclei of male and female rats. Rats were gonadectomized at least 1 week and not more than 3 weeks before assays were performed. Males and females were assayed together and were matched for age and length of time after gonadectomy. Steroid receptor levels were measured in hypothalamic nuclei by the specific binding to cytosol fractions of ³H-oestradiol (a) and ³H-R5020, a synthetic progestin (b). Tissue from hypothalamic nuclei was homogenized in 240 μ l of TEGD buffer (10 mM Tris-HCl, 5 mM EDTA, 10% glycerol and 1 mM dithiothreitol pH 7.4). The cytosol fraction of the homogenate was obtained by centrifugation in a Beckman Airfuge at 100,000g for 20 min. 100 μ l of cytosol were added to 50 μ l of TEGD buffer containing ³H-steroid plus unlabelled competitors. A 100- μ l aliquot of the incubate was applied to a Sephadex LH-20 column for gel filtration. The column was eluted with 400 μ l TEG buffer (TEGD buffer without dithiothreitol) 30 min after the sample was applied. The radioactivity in the eluate was measured by liquid scintillation counting at 34% efficiency. The level of nonspecific binding corresponded to no more than 25% of the total binding. Progestin receptor measurements were made in rats given oestradiol benzoate. Possible sex differences in non-oestrogen-induced progestin receptors were not examined because most of the neural actions of progesterone in females require oestrogen priming, and either require or are strongly correlated with oestrogen-induced progestin receptors³. Values are expressed as concentration of steroid ($\times 10^{-15}$ mol) specifically bound per mg protein: $n = 9$ for paired comparisons of oestrogen receptor levels in males and females; $n = 6$ for paired comparisons of progestin receptor levels. Significant sex differences were seen in oestrogen receptor levels in the medial preoptic area ($P = 0.045$, 2-tailed paired t comparison), in progestin receptors in the periventricular preoptic region ($P = 0.037$) and in the ventromedial nucleus ($P = 0.003$). There were no other significant sex differences in receptor content ($P > 0.120$ for all nuclei, 2-tailed paired t -test). NST, bed nucleus of the stria terminalis; MPOA, medial preoptic area; PVPOA, periventricular region of the preoptic area; PVAH, periventricular portion of the anterior hypothalamic nucleus; VMN, ventromedial nucleus; ARC, arcuate nucleus.

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Table 1 Location and protein content of hypothalamic nuclei

Region	Stereotaxic location	Protein (mg)	
		Male	Female
Bed nucleus stria terminalis	A7,190–6,290 μm	0.024 $\pm$ 0.001	0.026 $\pm$ 0.001
Medial preoptic nucleus	A7,190–6,590 μm	0.021 $\pm$ 0.001	0.020 $\pm$ 0.001
Periventricular preoptic nucleus	A7,190–6,590 μm	0.012 $\pm$ 0.001	0.012 $\pm$ 0.001
Periventricular anterior hypothalamic nucleus	A6,590–5,690 μm	0.018 $\pm$ 0.002	0.017 $\pm$ 0.001
Ventromedial nucleus	A4,890–3,690 μm	0.042 $\pm$ 0.002	0.045 $\pm$ 0.002
Arcuate nucleus	A4,890–3,690 μm	0.037 $\pm$ 0.003	0.035 $\pm$ 0.003

Nuclei were removed from frozen 300- μm thick sections of male and female rat brain, according to the procedure of Palkovits¹⁰, using a 500 or 1,000 μm diameter stainless steel cannula. The medial preoptic and ventromedial nuclei were removed with bilateral 1,000 μm punches. The periventricular anterior hypothalamic nucleus was taken with a single 1,000 μm punch, placed over the dense area of ³H-OE₂ binding described in the autoradiograms of Pfaff¹⁶. The bed nucleus of the stria terminalis was dissected with bilateral 500 μm punches above and below the decussation of the anterior commissure, and with bilateral 1,000 μm punches caudal to this. The periventricular preoptic region and the arcuate nucleus were removed with a small scalpel. The locations of nuclei are taken from the stereotaxic atlas of König and Klippel²¹. The protein values are the mean $\pm$ s.e.m. of the amount of cytosol protein applied to Sephadex LH-20 columns for steroid receptor measurements (see text for details). Protein was measured by the method of Bradford¹⁷; $n = 15$ for both males and females.

per mg protein). The level of progesterin receptor in the male ventromedial nucleus after OB treatment was comparable to unstimulated (non-OB-treated) levels in the female ventromedial nucleus¹⁵. The ventromedial nucleus of the hypothalamus may be the principal target site in rat brain for the activation of proceptive and receptive sexual behaviour by progesterone³. The periventricular region of the preoptic area also showed a significantly lower level of progesterin receptor in males than in females (25 as opposed to 50×10^{-15} mol per mg protein). There were no other significant sex differences in oestrogen or progesterin receptor content among the six nuclei examined (Fig. 1).

The sex differences in certain regions having a high content of oestrogen and progesterin receptors could explain particular sex differences in sensitivities to gonadal steroids. The substantially lower level of oestrogen receptors in male medial preoptic nucleus might explain in part why luteinizing hormone (LH) release in male rats is not stimulated by oestrogen. The local implantation of oestrogen into the medial preoptic area will stimulate LH release, while ablation of the medial preoptic nucleus blocks the LH surge¹⁶. The very low level of progesterin receptors in the ventromedial nucleus after oestrogen treatment may explain the inability of progesterone to produce proceptive behaviours and increase receptivity in oestrogen-primed males³. The implantation of nanogramme amounts of progesterone into the ventromedial nucleus will facilitate lordosis and activate sexual proceptivity in oestrogen-primed females¹⁸. The same small quantities of progesterone have no effect on sexual behaviour when applied to other hypothalamic nuclei. The effects of progesterone can also be blocked by the local application of a protein synthesis inhibitor to the ventromedial hypothalamus¹⁹. There is little information on the possible functions of the high level of progesterin receptors in the periventricular preoptic area, thus the significance of the large sex difference in this region is unclear. It is possible that sex differences also exist in brain regions with a low to moderate amount of gonadal steroid receptors. These regions were not examined in the present study.

Sex differences in oestrogen and progesterin receptors do not necessarily occur in the same nuclei (Fig. 1). In the medial preoptic area, the difference in oestrogen receptor content is not accompanied by a sex difference in progesterin receptors. It may be that the subset of oestrogen-sensitive cells which make progesterin receptors are not sexually dimorphic. Conversely, the sex differences in progesterin receptors in the ventromedial and periventricular preoptic nuclei are not accompanied by a sex difference in oestrogen receptors; it is possible that the process of progesterin receptor induction has been suppressed in these nuclei during sexual differentiation.

In previous studies on whole regions of the hypothalamus and preoptic area^{3,4}, we found no sex differences in cytoplasmic oestrogen and progesterin receptors. The amount of steroid receptor in an individual nucleus is a small part of the total amount of receptor in a hypothalamic tissue block, thus it is

not surprising that sex differences were not detected in these studies. Our measurements of cytoplasmic receptor levels do not exclude the possibility that sex differences also exist in the translocation of gonadal steroid receptors to cell nuclei. There are several reports of sex differences apparent in whole hypothalamic blocks of rat brain, when the nuclear retention of ³H-oestradiol is measured^{5,7}, although other laboratories have found similar levels of nuclear uptake in males and females^{8,9}. The measurement of cell nuclear receptor levels for gonadal steroids in microdissected brain regions would clarify the existence of sex differences in nuclear uptake. Note also that in order to minimize binding to low-affinity progesterin sites¹², we used a concentration of ³H-R5020 that occupies only 70–80% of the high-affinity component of the binding. Until we are able to perform binding isotherms on the limited amount of receptor in a punch-out sample, it remains possible, although unlikely, that the rather large male–female differences in ³H-R5020 binding represent differences in affinity rather than differences in concentration.

It is possible that regionally localized sex differences in gonadal steroid receptors exist in the brains of many species. Using quantitative autoradiography, Arnold and Saltiel²⁰ have found sex differences in the nuclear uptake of androgens in the song control nuclei of zebra finch brain. Their results and those presented here indicate that sex differences in neural receptors for gonadal steroids could be a general consequence of sexual differentiation, and may even exist in steroid-concentrating regions of human brain.

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A circulating inhibitor of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ associated with essential hypertension

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The aetiology of essential hypertension, a disease prevalent in cultured societies, is unknown. However, much evidence suggests that abnormal sodium metabolism has a critical role—this has led to the hypothesis that an increase in the circulating concentration of an inhibitor of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ is responsible for the increased peripheral vascular resistance in essential hypertension¹. Evidence for relatively high levels of a Na^+ pump inhibitor in essential hypertension has come from bioassay and cytochemical assays of plasma and urine from normotensive and hypertensive individuals^{2,3}. There is also evidence for increased plasma levels of a Na^+ pump inhibitor in some animal models (for example, renal and deoxycorticosterone acetate (DOCA) models) of hypertension⁴. Nevertheless, direct biochemical determination of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ inhibition by this substance has not yet been reported. We demonstrate here, with a kinetic $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ assay, a highly significant correlation between levels of a plasma inhibitor of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ activity and mean arterial blood pressure (MAP) in normotensive and hypertensive individuals. These data provide evidence for the involvement of a circulating Na^+ pump inhibitor in the genesis of essential hypertension. Moreover, our assay methods may be useful for the isolation and characterization of this inhibitor.

We have tested the effect of deproteinized blood plasma samples on the activity of a partially purified canine kidney $(\text{Na}^+ + \text{K}^+)\text{ATPase}$. In our assay, the regeneration of enzymatically hydrolysed ATP is coupled to the oxidation of NADH so that the action of various inhibitors can be monitored by continuously recording the absorbance of NADH at 340 nm. As we are concerned here with the activity of a humoral inhibitor of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$, we compared it with the behaviour of two well characterized inhibitors of the ATPase; Fig. 1a shows the features of inhibition by ouabain. This cardiotonic steroid had no significant effect on the reaction for the first 30 s, and thereafter caused a progressive, dose-dependent increase in inhibition⁵. Steady-state levels of inhibition were not achieved in the short time course presented. Similarly, the inhibition by vanadate (Fig. 1b) increased with time⁶, but because of the much faster association rate constant for binding, an effect of vanadate was apparent during the first few seconds of the reaction, especially at higher concentrations. By measuring the steady-state levels of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ activity in the presence of various vanadate concentrations, we estimate a K_i of 0.9 μM , in agreement with published values^{7,8}.

Figure 1c shows the kinetic behaviour of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ assayed in the presence of a saline solution (control) and deproteinized plasma samples derived from one normotensive individual, and from a patient with essential hypertension. In each case, about 1.5 min were required to reach a steady state of enzyme activity, which then lasted for at least 10 min (not shown). The normotensive NT plasma produced (in this

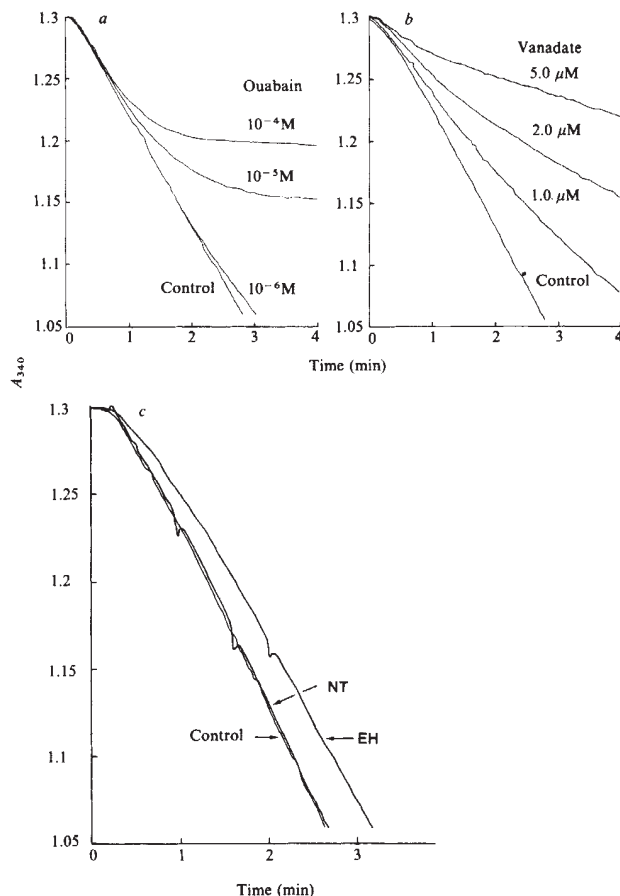


Fig. 1 Inhibition of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ activity by ouabain (a), vanadate (b), and plasma samples (c). Canine kidney $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ was obtained from Sigma (specific activity 5–6 μmol per min per mg protein). This preparation also contained an ouabain-insensitive Mg^{2+} -ATPase activity which was less than 2% of the total steady-state ATPase activity. The ATPase assay consisted of 400 μl ATPase cocktail to which 580 μl of H_2O were added. Final reaction conditions were (in 1.0 ml) as follows (in mM or appropriate units): KCl 20; NaCl 100; MgSO_4 4.5; EGTA 5; Na_2ATP 3; phosphoenolpyruvate tricyclohexylammonium (PEP), 1.2; NADH, 0.25; TES-Tris, 40, pH 7.4; lactate dehydrogenase (LDH), 1.2 U; pyruvate kinase (PK), 1.2 U. ATP, PEP, NADH, LDH and PK were from Boehringer Mannheim Biochemicals; both enzymes were obtained as glycerol suspensions. $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ was prepared for assay by suspension in 500 μM Na_4EDTA , 10 mM trimethylaminoethanesulphonic acid (TES)-Tris pH 7.4, at a protein concentration of 150 $\mu\text{g ml}^{-1}$. The resuspended enzyme was incubated at 25 °C for 3 h, and then stored on ice; the activity of the ATPase thus prepared does not change by more than 1% during the following 20 h on ice. The assay was initiated by rapid addition of 20 μl of ATPase suspension (3 μg protein) to 980 μl of reaction cocktail in a 1.0-ml cuvette at 37 °C, and the decrease in A_{340} due to NADH oxidation ($\epsilon = 6.22 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$) was continuously recorded, as illustrated in the examples shown here. a, Ouabain (1- μl aliquots of stock solutions prepared in dimethyl sulphoxide, DMSO) was pre-added to the reaction cocktail to give the final concentrations indicated. Controls contained appropriate quantities of DMSO. b, Vanadate, as Na_2VO_4 , was freshly prepared in H_2O and similarly pre-added to the reaction. Neither ouabain nor vanadate, at the concentrations shown, had any significant effect on the efficacy of the regenerating system, as determined by inspection of rates of NADH oxidation in response to addition of 10–50 μM ADP. The activity of the regenerating system was found to be over 70-fold greater than that of the total ATPase activity in these conditions. c, Effect of two individual plasma samples on the ATPase activity. Conditions were similar to those for a and b except that the assay cocktail contained (in mM): MgSO_4 , 9; and CaCl_2 , 2. 500 μl of H_2O were replaced by an equivalent volume of the following composition (in mM): KCl, 2.1; NaCl, 120; NaHCO_3 , 26; CaSO_4 , 2.5; K_2HPO_4 , 1.2; MgSO_4 , 1.2; EGTA, 3.4; EDTA, 2.8; and 800 $\mu\text{g ml}^{-1}$ bovine serum albumin. The EDTA and EGTA were included in this solution to control for their presence as anticoagulants in the plasma samples; see Fig. 2 legend for details. All the curves shown were traced from original records. Per cent inhibition was calculated as:

$$100 - \left(\frac{\text{sample } \Delta A_{340} \text{ min}^{-1}}{\text{control } \Delta A_{340} \text{ min}^{-1}} \right) \times 100.$$

example) no inhibition of activity compared with the control. In contrast, the hypertensive sample produced a 14% inhibition of steady-state activity which remained constant for the next 10 min of the reaction. This result demonstrates the presence of an increased level of an inhibitor of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ in hypertensive plasma that has a rapid onset of action in contrast to ouabain and vanadate.

This technique (see Fig. 1 legend) was used to assay plasma samples from 20 normotensive individuals and 26 patients with essential hypertension. A significant correlation between MAP and $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ inhibition was found when the hypertensive group alone was analysed ($r = 0.46$, $P < 0.01$); no significant correlation was found for the normotensive group. However, inclusion of the normotensives in the overall analysis (as illustrated) resulted in greater correlation coefficient and significance values ($r = 0.73$, $P < 0.0005$). Slightly smaller correlation coefficients ($r = 0.69$ and 0.68 , respectively), but similar P values, were obtained when the data from blacks and whites were evaluated separately. The correlation was similar ($r = 0.77$, $P < 0.0005$) when diastolic pressure was used instead of MAP. There was no significant correlation between $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ inhibition and patient age, perhaps because we attempted to age-match normotensives and hypertensives. The absence of females from the hypertensive group precludes an evaluation of the role of sex. One of the previously diagnosed hypertensive patients was normotensive on the day of blood withdrawal (MAP = 91 mm Hg); plasma samples from this individual exhibited a low ($< 4\%$) $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ inhibition. We have not yet obtained samples from patients with secondary forms of hypertension, therefore we cannot comment on the applicability of this assay as a diagnostic tool to discriminate between essential hypertension and secondary hypertension.

The maximal inhibition observed in this study was 14%—this is an underestimation of the inhibition expected *in vivo* because

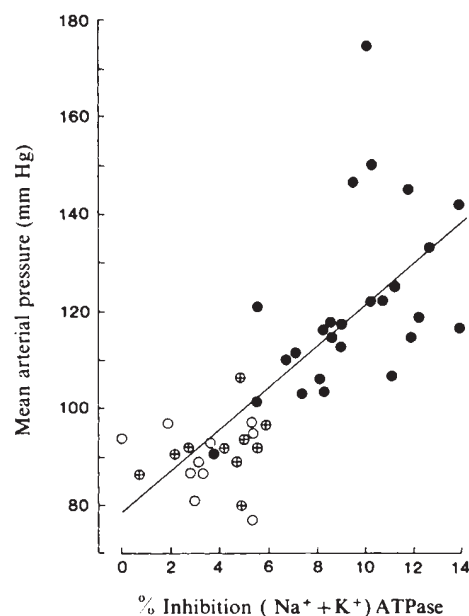
the plasma samples were diluted twofold in our assay. It seems reasonable to conclude that the apparent inhibitory activity represents an effect on $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ *per se*, because the contribution of ouabain-insensitive Mg^{2+} -ATPase to the total steady-state hydrolysis of ATP is less than 2% in these conditions.

As our $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ preparation consisted of unsealed membrane sheets⁹, we were unable to distinguish between an external and an internal site of action for the inhibitor. The data of Pamnani *et al.*⁴ on experimental models of hypertension, and those of Poston *et al.*³ on essential hypertension, indicate that the inhibitor acts at an external site, assuming (1) that the inhibitor does not cross the plasma membrane quickly, and (2) that our inhibitor is the same as theirs. Our kinetic data imply that the circulating inhibitor must bind much more rapidly than ouabain.

Figure 3 incorporates further data on red cell Na^+ levels and digoxin-like immunoreactivity in these plasma samples. The mean essential hypertensive MAP was more than two standard deviations above the mean normotensive MAP. Mean % inhibition of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ was significantly elevated in the EH group, as was intracellular erythrocyte Na^+ content.

The plasma samples were tested for digoxin-like immunoreactivity, following the report of an endogenous digoxin-like material in the plasma of volume-expanded dogs¹⁰ and hypertensive monkeys¹¹. This substance has been postulated to be an endogenous ligand for the cardiac glycoside binding site of the Na^+ pump. The action of such a substance may therefore be related to inhibition of Na^+ transport and elevation of cellular Na^+ as described in essential hypertension^{12,13}. However, as illustrated in Fig. 3, we were unable to demonstrate a significant elevation of digoxin-like immunoreactivity in the plasma samples that inhibited the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$. Furthermore, there was no correlation between the two assays, which suggests that the $(\text{Na}^+ +$

Fig. 2 Relationship between mean arterial blood pressure and inhibition of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ by plasma samples from normotensive and hypertensive individuals: 20 normal subjects (age 34 ± 14 yr, range 21–66 yr) and 26 patients (age 49 ± 11 yr, range 32–67 yr) suffering from essential hypertension were studied. All the hypertensive subjects were males; 18 were black. Twelve of the normotensives were males, and six were black. None of the normotensives had known endocrine abnormalities, and none of the hypertensive patients showed any evidence of end-organ damage or serious intercurrent disease. All patients with essential hypertension had blood pressures higher than the 95th percentile for age and sex on at least three previous office visits, and had received no drugs for at least 2 weeks before the study. Thirty-six individuals, including all the patients with hypertension, were characterized endocrinologically using integrated hormone profiling based on the principle of continuous 6-h blood withdrawal^{23,24}. The rationale for this collection protocol has been discussed elsewhere²³. Plasma samples were collected continuously for 6-h periods using an indwelling non-thrombogenic catheter connected to a constant withdrawal pump. A sample (10 ml) was collected every 30 min into glass tubes containing (final concentrations) 2.8 mM EDTA, 3.4 mM EGTA and 2.6 mM glutathione on ice. Cells and plasma were separated by immediate centrifugation and the plasma was stored on ice. After the 6-h withdrawal, aliquots of the 30-min samples were combined to give a 6-h integrated pool. Plasma renin activity, aldosterone, cortisol, noradrenaline and adrenaline were measured as described elsewhere^{23,24}, no significant correlation was found between levels of these substances and blood pressure (data not shown). Systolic and diastolic blood pressures were measured at hourly intervals during blood collections, and the results were averaged. Mean arterial pressure was calculated as: $\text{MAP} = 0.33 \times \text{pulse pressure} + \text{diastolic pressure}$. Plasma samples were analysed for Na^+ and K^+ (by flame photometry), inorganic phosphate²⁵ and protein content²⁶, and were stored at -20°C . Plasma was subsequently deproteinized by acidification to pH 5.5 with 1.0 M HCl and boiled for 2 min. The resultant slurry was centrifuged at 30,000g for 30 min at 4°C , and the clear supernatant removed for electrolyte and protein content determinations as before. The degree of protein removal was typically 99%, giving a final protein content of $800 \pm 180 \mu\text{g ml}^{-1}$ in the supernatants. The deproteinized samples were then assayed for $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ inhibitory activity as described in Fig. 1c legend, and the remainder was lyophilized for use in the digoxin radioimmunoassay (RIA) (see Fig. 3). The order of assay samples was randomized to avoid bias. The average coefficient of variance for repeated estimations on the same samples was typically 1% or better. The final electrolyte composition of the assay cocktail varied because of the addition of plasma samples. Thus, the final assay conditions varied as follows: (in mM except as stated) K^+ , 22–23; Na^+ , 170–175; total Ca^{2+} , 3.25–4.5; free Ca^{2+} , $< 10^{-7}$ M; total Mg^{2+} , 9.5–9.6; free Mg^{2+} , 2.1–2.8; total EGTA, 6.7; free EGTA, 2.2–100 μM ; total EDTA, 1.4–2.1; free EDTA, $< 10^{-8}$ M; total ATP, 3; free ATP, $< 10^{-7}$ M; PO_4 , 0.4–0.6. Surprisingly, we found that controlled variation of K^+ (20–30 mM), Na^+ (100–200 mM), free EGTA (1–500 μM), free Mg^{2+} (2–2.8 mM), and PO_4 (0.4–0.6 mM) gave no more than a 2% change in the steady-state activity of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$. High concentrations ($> 10^{-4}$ M) of free Ca^{2+} and ATP are both potent inhibitors of the ATPase but, due to the presence of excess EGTA and Mg^{2+} , respectively, they are reduced to non-interfering levels in our assay. In addition, the excess EGTA also serves to chelate other trace elements that may interfere with the assay. Given these and other considerations, we estimate that the cumulative error for each determination of ATPase activity was $\sim 3\%$. This uncertainty and the variability of blood pressure could contribute to the scatter observed. ●, ⊕ Correspond to 6-h integrated samples collected from patients with essential hypertension and from normotensives, respectively. ○, Discrete single samples collected from normotensives. The regression line was fitted by the least-squares method; the correlation between MAP and % inhibition is highly significant ($r = 0.73$, $P < 0.0005$).



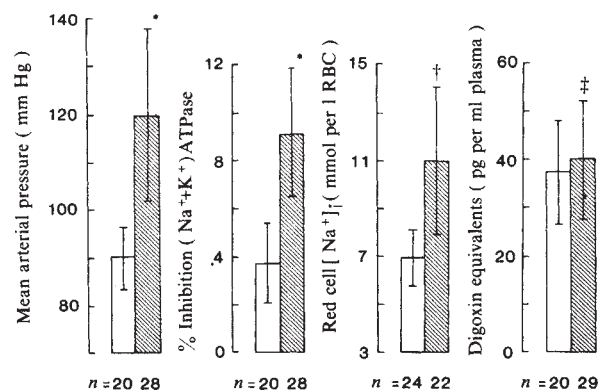


Fig. 3 MAP, % (Na⁺ + K⁺)ATPase inhibition, intracellular red blood cell (RBC) Na⁺ content and plasma digoxin equivalents in normotensive (open bars) and hypertensive (cross-hatched bars) individuals. Determination of MAP and inhibition of (Na⁺ + K⁺)ATPase are described in Fig. 2 legend. Red cell Na⁺ content was determined on freshly collected cells by atomic absorption spectroscopy after three washes of the cells in isotonic MgCl₂, determination of haematocrit and lysis of cells in deionized H₂O. Subsequent dilutions were performed in CsCl. For technical reasons, 34 of the red cell Na⁺ determinations were performed on cells from individuals other than those tested for (Na⁺ + K⁺)ATPase inhibition and digoxin-like immunoreactivity. However, all the blood samples were obtained from individuals in the same populations as those identified for study. Plasma digoxin equivalents were determined by digoxin RIA (NEN) essentially as described by Gruber *et al.*¹⁰. Deproteinized plasma samples were lyophilized and resuspended to 10× their original concentration in H₂O and assayed for their ability to compete with iodinated digoxin for a digoxin-specific antibody. Some samples were seeded with digoxin to ensure that the antibody was not affected by the increased electrolyte content of the assay. The data presented are corrected for concentration and dilution factors incurred during sample preparation, and are described as pg digoxin equivalents per ml plasma. None of the individuals had received cardiac glycosides. Values shown are mean ± s.d. of the indicated number of samples. Significance of the differences in the means was determined by Student's *t*-test: **P* < 0.0005; +*P* < 0.005; ± not significant (*P* > 0.1).

observations imply that, even in normal circumstances, the activity of Na⁺ pumps may be directly modulated *in vivo* by humoral substances.

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Nonspecific inhibitor released by T acceptor cells reduces the production of interleukin-2

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Mice exposed to contact sensitizing agents such as oxazolone (ox) or picryl (pic) derivatives develop specific immunological suppression to these substances which results from the activation of a circuit of T suppressor cells^{1–3}. However, one of the final immunological events in the T suppressor cell circuit is the release of a nonspecific, H-2 unrestricted inhibitor by I-J⁺, Lyt-2⁺ cyclophosphamide-sensitive T acceptor cells (Tacc)^{3–7}. This nonspecific inhibitor (nsINH) can block the passive transfer of contact sensitivity and depress DNA synthesis by lymphocytes exposed to mitogens and alloantigens but the molecular mechanism is poorly understood. We provide evidence here that the production of interleukin-2 (IL-2)^{8–15} by stimulated lymphoid cells is suppressed when the cells have been exposed to nsINH.

The T suppressor cell circuit, which has an important role in the modulation of immune responses to ox and pic derivatives, involves Lyt-1⁺2⁺ hapten-specific T suppressor cells producing hapten-specific T suppressor factor (TsF), which arms the Tacc; the armed Tacc population then liberates nsINH when exposed to hapten-modified (oxazonated or picrylated) lymphoid cells corresponding in hapten specificity to TsF and bearing the same I-J subregion as the source of TsF^{3–7}. Our initial observation was that nsINH diminishes proliferative responses to con-

K⁺)ATPase inhibitor we describe and the digoxin-like substance may not be related.

We have previously discussed the manner in which inhibition of Na⁺ transport may lead to a rise in blood pressure^{1,14,15}. Inhibition of Na⁺ pumps in vascular tissue by ouabain results in increased resting tone¹⁶ and enhanced reactivity to catecholamines¹⁶. As an increase in intracellular free Ca²⁺ is the immediate trigger for concentration in vascular smooth muscle¹⁷, it seems reasonable to suppose that inhibition of Na⁺ pumps leads to a net accumulation or redistribution of intracellular Ca²⁺. This phenomenon may occur as a consequence of a Na⁺–Ca²⁺ exchange mechanism at the vascular smooth muscle cell plasma membrane^{1,14,15}, whereby the increase in intracellular Na⁺ by Na⁺ pump inhibition leads to a net uptake of Ca²⁺ and, thus, to an increase in peripheral vascular resistance—the hallmark of the hypertensive process¹⁸. In addition, inhibition of Na⁺ pumps in sympathetic nerve terminals may enhance catecholamine release¹⁹ and reduce re-uptake²⁰, and thus contribute to the maintenance of elevated vascular smooth muscle tone¹⁵.

The sequence of events leading to the elevated levels of (Na⁺ + K⁺)ATPase inhibitor in the plasma may be the result of a congenital (or, in some types of secondary hypertension, acquired) defect in the renal natriuretic capability in individuals prone to develop hypertension²¹. Excessive Na⁺ ingestion by these individuals would result in Na⁺ and water retention and the tendency towards expansion of the extracellular fluid volume unless adequate compensatory physiological mechanisms are brought into play. Our data support the view that one such compensatory mechanism may be increased secretion of an inhibitor of (Na⁺ + K⁺)ATPase that enhances Na⁺ excretion (that is, a natriuretic hormone²²). Increased circulating amounts of this substance may induce hypertension as a side effect¹. In addition to their possible significance for pathophysiology, our

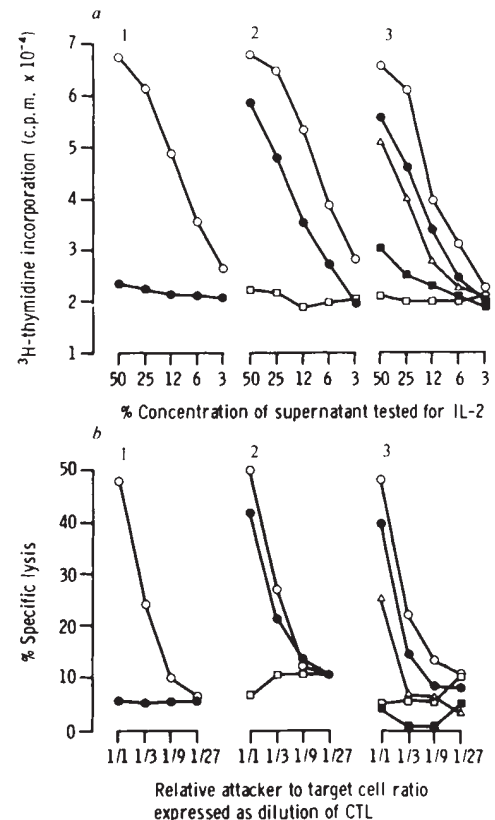
Fig. 1 Reduced IL-2 activity in supernatants of Con A-activated mouse spleen cells exposed to nsINH. A lymphoid cell suspension was prepared by pressing BALB/c spleens through a fine mesh screen into RPMI 1640 medium. Erythrocytes were lysed using Boyle's solution²⁷. The lymphoid cells were mixed with various concentrations of the nsINH supernatant or with 100% of the control supernatants (10^7 cells ml^{-1}); Con A ($5 \mu\text{g ml}^{-1}$) was added and the suspension was distributed in 4-ml aliquots into 12-well flat bottom Linbro tissue culture plates (Flow). Similarly, 4-ml aliquots of the same cells (10^7 cells ml^{-1}) were resuspended in RPMI 1640 medium with (positive assay control) or without (negative assay control) Con A ($5 \mu\text{g ml}^{-1}$), and distributed into the plates. After 4 h incubation, the supernatant was carefully removed and discarded. Fresh serum-free RPMI 1640 medium (without Con A) was added and the supernatant was collected after a further incubation for 20 h and tested for IL-2 activity in two different assays. The presence of IL-2 was measured initially using 4 day Con A blast cells essentially following ref. 14 (Fig. 1a). BALB/c Con A blast cells (10^6 cells ml^{-1}) were cultured in 0.2 ml supplemented RPMI 1640 medium in the presence of various concentrations of the supernatants to be tested for IL-2 activity. Cultures were set up in quadruplicate in 96-well Titertek tissue culture plates. Five hours before collection, each well was pulsed with $1 \mu\text{Ci}$ [$\text{Me-}^3\text{H}$]thymidine. The cultures were collected after 25 h and tritiated thymidine incorporation was determined. In a, the supernatants tested for the IL-2 activity were: panel 1: supernatant from untreated spleen cells, the negative assay control ($\bullet$), supernatant from Con A-activated spleen cells, the positive assay control ($\circ$); panel 2: supernatants from Con A-activated spleen cells exposed to a 100% nsINH supernatant ($\square$) or to 100% control supernatants prepared with the omission of either antigen ($\diamond$) or TsF ($\blacklozenge$) (see Table 1); panel 3: supernatants from Con A-activated spleen cells exposed to different concentrations of the nsINH supernatant—50% ($\square$), 25% ($\blacksquare$), 12% ($\triangle$), 6% ($\bullet$), 3% ($\circ$). The abscissa indicates the concentration of the supernatants tested for IL-2. The ordinate indicates the IL-2 activity as measured by the tritiated thymidine incorporation by Con A blasts. Con A blasts incubated in culture medium only for 25 h incorporated tritiated thymidine to a similar extent as the negative assay control ($19,555 \pm 2,721$ c.p.m.). In b, the same supernatants were assayed for IL-2 activity by their capacity to sustain the growth and function of cultured CTL^{9,16}. The following modification of the original method described in ref. 16 was used. Female CBA (H-2^k) spleen cells from untreated mice (4×10^6 cells ml^{-1}) were co-cultured with irradiated (2,000 R, ^{60}Co source) NZB (H-2^d) stimulator lymphoid cells (2×10^6 cells ml^{-1}) in supplemented RPMI 1640 medium with 5×10^{-5} 2-mercaptoethanol. Cultures were seeded in 12-well flat bottom Linbro tissue culture plates in a total volume of 4 ml per well. After 5 days, dead cells were removed by centrifugation over Ficoll/Isopaque²⁸. Live cells (0.25×10^6 cells per ml) were co-cultured with fresh irradiated NZB stimulator lymphoid cells (10^6 cells per ml) for another 2 days. The cells were then collected, purified over Isopaque/Ficoll²⁸ and plated in 24-well flat bottom Linbro tissue culture plates (Flow). Cells (2×10^4 cells ml^{-1}) were incubated in various concentrations of supernatants to be tested for the IL-2 activity or in culture medium for 3 days. For the cytotoxicity assay, the cells were washed once and resuspended in 0.5 ml supplemented RPMI 1640 medium; 0.1-ml triplicates of four threefold dilutions were set up in 96-well U-bottom Microtiter plates (Sterilin) and incubated in a total volume of 0.2 ml with P815 (H-2^d) target cells (3×10^3 cells per well) labelled with $\text{Na}_2^{51}\text{CrO}_4$ (specific activity: 100–400 mCi per mg Cr, Amersham, UK). The Cr-release was stopped by centrifugation after 3 h incubation by centrifugation (300g, 10 min, 4°C). The Cr-release c.p.m. were determined by counting a 100- μl supernatant from each well in a gamma counter.

$$\% \text{ Specific lysis} = \frac{\text{experimental c.p.m.} - \text{medium control c.p.m.}}{\text{maximum release c.p.m.} - \text{medium control c.p.m.}} \times 100$$

Medium control c.p.m. were obtained from 100- μl supernatant aliquots taken from wells containing radiolabelled target cells only. Maximum release c.p.m. were obtained from wells containing radiolabelled target cells in 5% Triton X-100 (BDH). b Shows the cytotoxic potential of murine secondary CTL incubated for 3 days in the presence of a 25% concentration of the supernatants tested for the IL-2 activity and the panels are as in a. Relative attacker to target cell ratios are indicated on the abscissa and % specific lysis on the ordinate. Very similar results were obtained with other concentrations of the supernatants and the cytotoxic cells were shown to be T lymphocytes with monoclonal anti-Thy-1.2 antibody (Olac) and complement (unpublished data).

canavalin A (Con A) only when added less than 8 h after the proliferative stimulus^{3,7}. However, the effect of nsINH could be overcome by adding exogenous IL-2 but not by increasing the concentration of Con A. These findings prompted investigation of the possibility that the effect of nsINH could be due to the relative lack of IL-2 in cultures of stimulated lymphoid cells which had been treated with nsINH. In the first set of experiments we prepared control supernatants and supernatants containing nsINH and tested them in the DNA synthesis assay (Table 1). The nsINH supernatant (50% concentration) of BALB/c origin depressed Con A-induced DNA synthesis to ~40% of the control value in syngeneic BALB/c (Table 1) or in allogeneic B10.A or CBA (data not shown) indicator cells, while control supernatants were inactive.

The second set of experiments examined the ability of nsINH supernatants to influence Con A-induced IL-2 production by lymphoid cells. BALB/c spleen cells were suspended in culture



medium or in 100% control supernatants or in different concentrations of a supernatant with nsINH activity and exposed to Con A. After a 4-h incubation, the culture fluids were removed and replaced with serum-free RPMI 1640 medium. The production of IL-2 by the cells was assessed by measuring the IL-2 activity in the culture medium after a further 20-h incubation using both proliferation¹⁴ and functional^{9,16} assays for IL-2 (Fig. 1). The results, confirmed in seven independent experiments, showed that the production of IL-2 by stimulated lymphoid cells was at least 15-fold lower as estimated by the biological assays for IL-2, when the cells were exposed to 25–100% concentration of nsINH supernatant. Moreover, the action of nsINH was nonspecific and H-2-unrestricted and was not limited to Con A-induced IL-2 production. Table 2 shows that nsINH of BALB/c or CBA origin inhibited the production of IL-2 in supernatants from Con A- or alloantigen-stimulated BALB/c or CBA spleen cells.

Table 1 Nonspecific inhibitor depresses the DNA synthetic response to Con A in spleen cells

Tacc (4-day ox spleen/lymph- node cells)	Preparation of supernatant		Additions to test culture	³ H-thymidine incorporation (c.p.m. $\pm$ s.d.)	% of Con A response
	TsF (anti-picryl)	Antigen (picrylated spleen cells)			
			—	57 $\pm$ 10	
			Con A (2 μ g ml ⁻¹)	45,800 $\pm$ 379	100 (standard)
+	+	—	Con A + 50% control supernatant (no antigen)	44,857 $\pm$ 2,017	98
+	—	+	Con A + 50% control supernatant (no TsF)	41,693 $\pm$ 4,970	91
+	+	+	Con A + 50% nsINH supernatant	17,489 $\pm$ 341	38

The nsINH is released by Tacc armed with TsF and triggered by antigen³⁻⁷. Six to nine-week-old female BALB/c mice were used throughout. The anti-picryl T suppressor factor (pic TsF) was produced by 48-h cultures of lymphoid cells from mice injected and then painted with picryl derivatives^{6,7,24}. The lymphoid cells (10⁷ cells per ml) were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ in supplemented RPMI 1640 medium (Flow). The supplemented RPMI 1640 medium contained 5% fetal calf serum (Flow), 2 $\times$ 10⁻³ M L-glutamine (Sigma), 1 $\times$ 10⁻³ M HEPES (Flow), 100 IU ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin (Glaxo). Nylon wool purified T cells²⁵ from spleen and lymph nodes taken 4 days after painting mice with oxazolone were used as the Tacc population^{6,7}. The spleen cells were picrylated by the method of Shearer²⁶. Control supernatants and supernatants containing nsINH were prepared following refs 6 and 7. In brief, the Tacc population was incubated in a supernatant containing pic TsF (25 $\times$ 10⁶ cells per ml) for 1 h and shaken occasionally. The armed Tacc were recovered by centrifugation and mixed with a third of their number of picrylated cells, centrifuged, resuspended in supplemented RPMI 1640 medium (25 $\times$ 10⁶ Tacc ml⁻¹) and incubated for 2.5 h. The suspension was centrifuged and the supernatant filtered through a 0.22- μ m Millex-GS filter (Millipore). Control supernatants were prepared by omitting either picrylated cells or pic TsF. These supernatants were tested in the DNA synthesis assay. Briefly, spleen cells from untreated mice (7.5 $\times$ 10⁵ cells ml⁻¹) were cultured in 0.2 ml of supplemented RPMI 1640 medium with 2 $\times$ 10⁻⁵ M 2-mercaptoethanol (type I, Sigma) and 2 μ g ml⁻¹ Con A (Pharmacia). (The batch of fetal calf serum was selected to be nonmitogenic for mouse cells so that DNA synthesis in cultures untreated with mitogens was less than 2% of DNA synthesis in mitogen-treated cultures.) Cultures were set up in quadruplicate in 96-well Titertek tissue culture plates (Flow). The final concentration of supernatants in the assay was 50%. Five hours before collection, each well was pulsed with 0.5 μ Ci [*Me*-³H]thymidine (specific activity: 5 Ci mM⁻¹, Cat. no. TRA.120, Amersham). The plates were collected after 72 h using a multicell cell collector (Scatron) and Titertek collector filters (Flow). Tritiated thymidine incorporation was determined by liquid scintillation counting in 2 ml Micellar Scintillator (Nuclear Enterprises).

It was possible that nsINH might act indirectly by inducing the release of an IL-2 inhibitor¹⁷, which could block the action of IL-2. To exclude this possibility, the IL-2 supernatant (the positive assay control in Fig. 1) and the supernatant from Con A-activated spleen cells exposed to nsINH were subject to preparative isoelectric focusing (Fig. 2). In the control supernatant, the IL-2 activity was recovered as a single peak in the pH range of 3.8 to 4.6; peak activity migrated with an isoelectric point of 4.1 in keeping with ref. 14. The results of Watson *et al.*¹⁸, who reported two distinct peaks (pI 4.3 and 4.9) of murine IL-2 activity, may reflect the known heterogeneity of IL-2 which is due in part to variable glycosylation. However, no IL-2 activity was recovered from the supernatant from Con A-activated cells exposed to nsINH. Moreover, the data in Table 3 show that inactive supernatant did not affect the ability of IL-2 to promote cell proliferation. It was concluded that nsINH reduced the production of IL-2 by lymphoid cells and did not induce an inhibitor of the action of IL-2. The nsINH was not nonspecifically toxic to lymphoid cells because the cell viability assessed by trypan blue dye exclusion of normal

unstimulated spleen cells was unaffected by the presence of nsINH. Moreover, the growth of lymphoid cell lines BW 5147 (ref. 19), EL4 (ref. 20) and P3-NSI/1-Ag4-1 (ref. 21) was not influenced by nsINH. Finally, nsINH had no effect on Con A-induced cell proliferation when added after the first 8 h of culture^{3,7} and its effect when added at the beginning of culture was abolished by exogenous IL-2. For instance, in an experiment in which spleen cells, stimulated by Con A, incorporated 94,963 $\pm$ 8,174 c.p.m. of tritiated thymidine on day 3, but only 25,771 $\pm$ 3,325 c.p.m. in the presence of 50% nsINH, cell proliferation was completely restored (96,008 $\pm$ 7,027 c.p.m.) by adding 20% supernatant containing exogenous IL-2.

Preliminary data suggested that the nsINH, which had an isoelectric point of 6.4–6.9 and a molecular weight of 40–60,000, did not influence the differentiation of primary or secondary cytotoxic T lymphocytes (CTL)^{3,7}. These data are compatible with the view that a distinct soluble factor but little or no IL-2 are required to induce differentiation of effector CTL from resting precursors^{22,23}. The present T suppressor cell circuit³⁻⁷ involving the liberation of nonspecific inhibitor of the

Table 2 The action of nonspecific inhibitor is not H-2-restricted

Origin of IL-2	IL-2 assay (c.p.m. $\pm$ s.d.)		
	Nonspecific inhibitor absent	Nonspecific inhibitor present (50% v/v)	
		Origin of the nonspecific inhibitor	
		BALB/c	CBA
Alloantigen-stimulated BALB/c	27,211 $\pm$ 3,185	5,824 $\pm$ 795	5,762 $\pm$ 823
Con A-stimulated BALB/c	45,895 $\pm$ 3,455	5,956 $\pm$ 1,491	6,167 $\pm$ 1,002
Alloantigen-stimulated CBA	29,286 $\pm$ 1,920	5,837 $\pm$ 921	5,626 $\pm$ 813
Con A-stimulated CBA	49,186 $\pm$ 3,696	6,170 $\pm$ 504	5,844 $\pm$ 879

Mixed lymphocyte cultures were set up as described elsewhere⁷. In brief, BALB/c or CBA spleen cells at a final concentration of 5 $\times$ 10⁶ cells ml⁻¹ were co-cultured with equal numbers of irradiated (2,000 R, ⁶⁰Co source) allogeneic stimulator CBA or BALB/c spleen cells in the absence or presence of nsINH. After a 3-day incubation, the supernatant was removed and replaced with fresh culture medium, which was collected after a further 1 day incubation and tested for IL-2 activity. The preparation of supernatants from Con A-stimulated spleen cell cultures and the testing of the IL-2 activity of the supernatants by their capacity to promote the proliferation of 4-day Con A blasts are described in the legend to Fig. 1. The data in Table 2 show IL-2 production as measured by the proliferation of 4-day Con A blasts incubated for 25 h in the presence of a 25% concentration of supernatant. Similar results were obtained with other concentrations (50, 12, 6%) of the supernatants. The incorporation of tritiated thymidine into Con A blasts incubated for 25 h in culture medium only was 5,407 $\pm$ 807 c.p.m. Alloantigen- or Con A-stimulated cells liberated approximately the same amounts of IL-2 as the same cells exposed to control supernatants prepared with the omission of either antigen or TsF (data not shown).

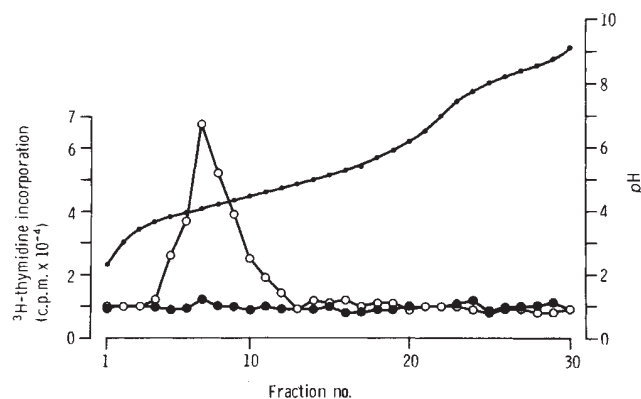


Fig. 2 IL-2 cannot be isolated by preparative isoelectric focusing from supernatants of Con A-activated spleen cell exposed to nsINH. Preparative isoelectric focusing was carried out in a flat-bed of Ultradex gel (LKB) using the LKB 2117 Multiphor Apparatus following refs 14 and 18. Briefly, a final concentration of 4% Pharmalyte ampholites (pH 3–10, Pharmacia) was added to samples, which were electrophoresed for 16 h at a constant power of 8 W. The gel was then sectioned into 30 slices, each slice was eluted with 2 ml H₂O and the pH was determined. Each sample was dialysed against phosphate-buffered saline, finally against RPMI 1640 medium and assayed for biological activity using Con A blast cells as described in Fig. 1 legend. The supernatants were from Con A-activated spleen cells (○) and from Con A-activated spleen cells exposed to a 100% supernatant containing nsINH (●). The pH curve is given for the supernatant from Con A-activated spleen cells. Fractions 4–15 of the second supernatant had the same pH and the difference between the pH of corresponding samples of remaining fractions was less than 0.2.

Table 3 Absence of an inhibitor of the action of IL-2 in a supernatant from Con A-activated spleen cells exposed to nsINH

Additions to test culture	³ H-thymidine incorporation (c.p.m. ± s.d.)	
	Medium	Supernatant
—	7,624 ± 230	7,508 ± 1,933
5% IL-2	21,001 ± 1,600	20,589 ± 763
10% IL-2	42,481 ± 1,885	42,230 ± 1,089
20% IL-2	57,381 ± 2,779	55,692 ± 1,207
30% IL-2	64,607 ± 4,577	63,792 ± 7,345
40% IL-2	79,773 ± 2,834	77,865 ± 774

Various final concentrations of an IL-2 supernatant (the positive control in the experiment described in Fig. 1) were prepared by diluting in either RPMI 1640 medium or in a supernatant, from Con A-activated spleen cells exposed to a 100% supernatant containing nsINH, in which IL-2 activity could not be detected directly (Fig. 1) or after preparative isoelectric focusing (Fig. 2). The dilutions were assayed for IL-2 activity by their capacity to promote the proliferation of 4-day Con A blasts using the technique described in the legend to Fig. 1.

production of IL-2 could provide a mechanism which limits the entry of new cells proliferating under the action of IL-2 into an immune response.

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Influenza A specific cytotoxic T-cell clones that do not recognize viral glycoproteins

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Since the demonstration that murine cytotoxic T cells generated during a secondary immune response to influenza A viruses were composed of cross-reactive and subtype-specific populations^{1–5}, it has become of interest to define the viral components responsible for each pattern of recognition. One approach is by the isolation of individual clones of cytotoxic T cells^{6,7} which can then be tested for recognition of natural or laboratory-induced virus variants of known genetic composition. We describe here a series of influenza A virus subtype-specific clones raised against the recombinant X31(H3N2)⁸ virus, which contains the haemagglutinin (HA) and neuraminidase (NA) from the 1968 virus, and all its remaining genes from the 1934 virus⁹. We show here that the viral gene responsible for the determinant recognized by these cytotoxic T-cell clones codes for neither the haemagglutinin nor neuraminidase glycoproteins, that it segregates independently of HA and NA during the formation of recombinant viruses, and that it undergoes independent genetic change.

The virus X31 (H3N2) is a recombinant between A/Aichi/1968 (H3N2) and A/PR/8/1934 (H1N1). Clones able to recognize the recombinant virus X31 (H3N2) but not A/USSR/90/77 (H1N1)-infected target cells are here called subtype-specific. The subtype-specific cytotoxic clones examined here all show either no or very little activity on A/USSR compared with X31, and were selected on this basis for closer inspection. In each experiment a cross-reactive clone, T6-11-5-1, or polyclonal line D, able to kill targets infected with any influenza A virus, served as a positive control, as different viruses tend to infect target cells with varying efficiency.

Figure 1 shows the spectrum of activity of clone A3.1. It reveals that target cells infected with A/PR/8/1934 are recognized but that A/Aichi/1968 is not, while the cross-reactive control kills both equally well. As A/PR/8/1934 contributes all the genes to X31 other than the HA and NA genes⁹, the

subtype-specific component being recognized seems not to be the haemagglutinin or neuraminidase surface glycoproteins. Figure 1 also shows that the virus component recognized underwent genetic change between 1943 and 1946 because the 1943 virus infected cells are killed as efficiently by A3.1 as by the cross-reactive control, whereas the 1946 and 1947 virus infected targets are not killed at all by A3.1 but are killed very efficiently by the cross-reactive control. The influenza B/Hong Kong/8/73 infected target acts as a specificity control for both cross-reactive and subtype-specific populations. Four other

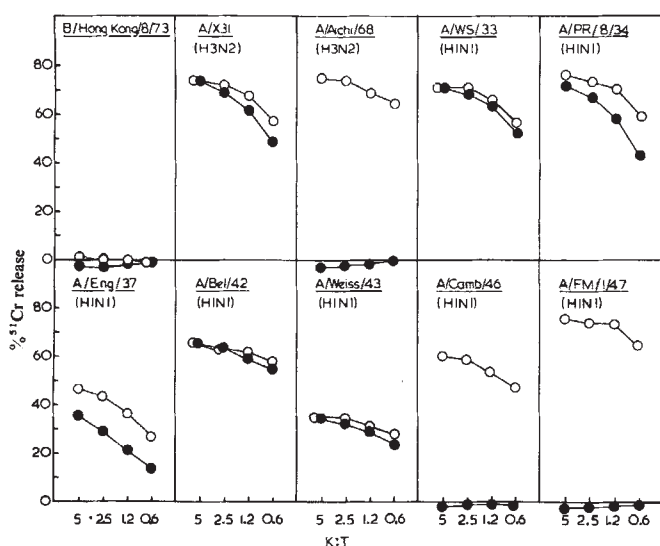


Fig. 1 Spectrum of activity of clone A3.1 on target cells infected with various influenza A viruses (●—●); line D effectors (○—○) act as cross-reactive control. Combined results from two experiments. T-cell clones: the derivation and maintenance of clone A3.1 and clones depicted in the other figures will be described in detail elsewhere (A.R.M.T. and P. M. Taylor, manuscript in preparation). Briefly, polyclonal cytotoxic lines were established by repeated stimulation *in vitro* of spleen cells from intranasally primed donor mice with X31 infected, irradiated, syngeneic spleen cells. After two stimulations growth factors were added each time as 20% v/v supernatant from concanavalin A pulsed rat spleen cells (CAPS/N). After at least two more stimulations *in vitro*, the cells were cloned by limiting dilution at least once at 0.5 cells per well onto an X31 infected syngeneic spleen cell feeder layer. Growing clones were transferred to 2-ml Costar wells after 7–14 days and maintained in Costars by stimulation every 3–5 days in the presence of 20% CAPS/N. The subtype-specific clones originated from X31 primed mice and were selected and maintained using X31 *in vitro*. Clones A3.1 and A3.6-5.4 were derived from the same cell line, B10.7, B8.3 and C2.2 from a different line. The cross-reactive control clone T6-11-5-1 and the polyclonal line D were derived from A/JAP/305/57 (H2N2) primed mice and were selected by X31 *in vitro*. All cells were grown in RPMI/1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, glutamine and 5×10^{-5} M 2-mercaptoethanol. All the subtype-specific clones described show H-2 restricted recognition, requiring the class I molecule D^b on the target cell surface²⁴. The cytotoxicity assay was performed as described previously²⁴. Cloned cytotoxic T cells were incubated, at the ratios indicated, with ⁵¹Cr-labelled target cells infected with the test virus (50–400 μ l infectious allantoic fluid per 10^7 cells per 90 min) in a 6-h assay. The per cent specific ⁵¹Cr released from lysed target cells was calculated as follows:

$$\frac{(\text{Release in presence of cytotoxic T cells} - \text{medium release})}{(\text{Triton release} - \text{medium release})} \times 100$$

All points measured in duplicate where four different killer:target ratios (K:T) were tested or quadruplicate where 1K:T was tested. Target cells were EL4 cells maintained as an ascitic tumour *in vivo*. Spontaneous ⁵¹Cr release in presence of medium (SR) = 13–34%.

clones (three from a separate culture), derived in the same way as A3.1, showed the same pattern (Table 1), killing A/PR/8/1934 targets but not A/Eng/42/1972 targets (which is antigenically similar to A/Aichi/1968).

One clone, A3.6-5.4, from the same culture as A3.1, was tested on a number of viruses of the H3N2 subtype isolated between 1968 and 1979 (Fig. 2). It was unable to kill targets infected with any of them, whereas the cross-reactive clone T6-11-5-1 was positive on all the A virus infected targets.

To be sure that the determinant from the 1934 virus being recognized was encoded by a gene other than HA or NA, we tested clones on targets infected with a variety of recombinant viruses produced by crossing post-1968 H3N2 viruses with either A/PR/8/1934 (H1N1) or A/Bel/1942 (H1N1) (Fig. 3). Of these X31, MRC11 and X47 are antigenically H3N2, MRC1 and Mem/Bel 102 are H3N1. As illustrated by clone A3-6-5-4 (Fig. 3) the X31 and MRC1 infected targets are killed, but not the MRC11, Memphis/Bel 102, or X47 targets, whereas the cross-reactive control kills all the targets infected with recombinant A viruses.

We have found that polyclonal cytotoxic T-cell lines originating from C57B/6 mice primed with X31 and maintained *in vitro* by stimulation with X31, initially show characteristic cross-reaction on all influenza A virus infected targets¹, but after several stimulations give rise to subtype-specific clones with high frequency (data not shown). There is thus, in the C57B/6 strain at least, a selection *in vitro* for the A virus subtype specific subset using this protocol. The clones discussed here are from such an *in vitro* selected population.

Previously, attention has naturally focused on the haemagglutinin molecule as a source of virus subtype-specific determinants recognized by B (refs 10, 11) and T cells¹², the more so because it has been shown by several workers that secondary cytotoxic T-cell responses induced *in vitro* with purified viral haemagglutinin show subtype-specificity, and the determinants responsible map to the HA gene when tested on appropriate recombinant viruses^{2,13}. Haemagglutinin-specific cytotoxic T cells must therefore exist.

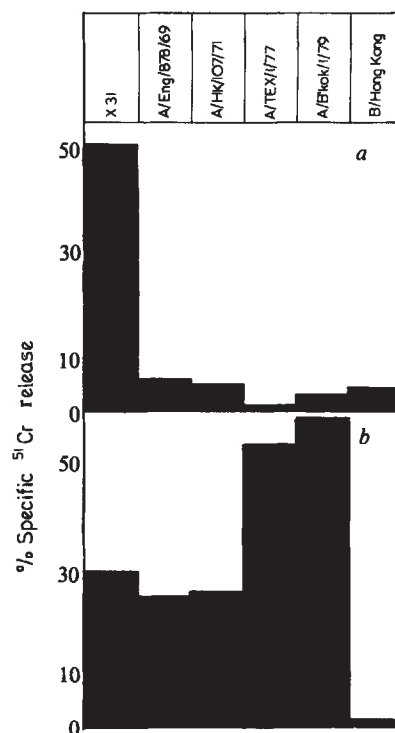


Fig. 2 a, Activity of clone A3.6-5.4 on target cells infected with post-1968 H3N2 influenza A viruses. b, Cross-reactive control clone T6-11-5-1. Combined results from two experiments. Thio-glycollate induced PEC as targets. SR = 25–42%.

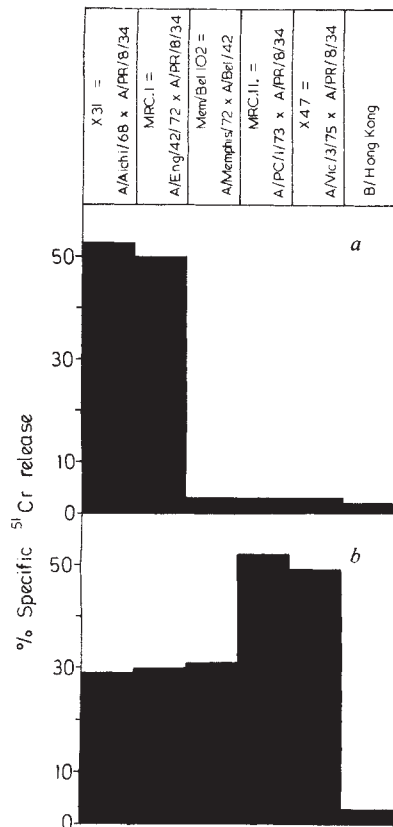


Fig. 3 *a*, Activity of clone A3.6-5.4 on recombinant viruses. *b*, Cross-reactive control clone T6-11-5-1. Combined results from two experiments. Thioglycollate induced PEC as targets. SR = 19–52%.

It was thus a surprise to find that all the T-cell clones isolated by us in the way described (see Fig. 1 legend) do not recognize either of the viral surface glycoproteins. The evidence for this is twofold. First, of the two viruses which contribute genes to X31, only the A/PR/8/1934 is recognized despite the fact that X31 does not receive its HA and NA genes from A/PR/8/1934. Second, when tested on a variety of recombinant viruses of known surface glycoprotein constitution, X31 (H3N2) and MRC1 (H3N1) are killed, but Mem/Bel 102 (H3N1), MRC11 (H3N2) and X47 (H3N2) are not (Fig. 3), showing that the viral gene responsible for recognition of infected target cells by clone A3.6-5.4 can segregate independently of the HA and NA genes during the formation of these five different recombinant viruses.

Braciale *et al.*⁷ describe a series of murine cytotoxic T-cell clones derived using a related protocol with A/JAP/305/1957 (H2N2) virus. A similarly high frequency of clones showed subtype specificity, and these could be divided into those able

to recognize targets infected only with 1957 H2N2 virus and those able to recognize both the 1957 and 1967 H2N2 viruses. Representatives of both groups when tested on recombinant viruses of known HA and NA composition seemed to be HA specific. However, it is possible that a non-HA gene responsible for recognition could have segregated by chance with the HA gene in the two recombinant viruses tested with a probability of 1/4.

Lamb *et al.*¹⁴ have described six human H3N2 influenza-subtype specific T-cell clones. All six gave a specific proliferative response only with either purified viral HA or NA, whereas five cross-reactive clones gave specific responses only with purified matrix protein (M) or nucleoprotein (NP). This result may, like our own, reflect the technique of *in vitro* selection used, and does not rule out subtype-specific determinants on viral components other than HA and NA.

The determinant we have defined with clone A3.1 undergoes genetic change, as shown in Fig. 1, between the 1943 and 1946 virus isolates. It is of interest that the timing of this change coincides with a major change in the antigenicity of the H1N1 nucleoprotein as shown by van Wyke *et al.*¹⁵ using monoclonal antibodies and Schild *et al.*¹⁶ using rabbit antisera to purified NP. Furthermore Schild *et al.*¹⁷ identified the origins of the NP in X31, X47 and MRC11, three of the recombinants we used. X31 is the only virus to receive the A/PR/8/1934 NP gene, and the only one of these three recognized by clone A3.6-5.4. However, from the results presented here none of the virus proteins other than the HA and NA glycoproteins can be excluded as the target for these clones. Also, although NP has been shown to be expressed on the surface of infected cells^{18,19}, there are also reports that NS1²⁰ and M protein²¹ can be detected on the surface of infected cells, albeit for the latter in very low quantities when compared with HA²².

A recent report²³ implicates the polymerase 3 gene as an absolute requirement for recognition by an isolated subtype-specific cytotoxic T-cell clone. This clone was derived by stimulation with A/PR/8/1934, and the recombinant A virus recognition data conclusively showed that neither the HA nor NA genes from A/PR/8/1934 were required. Thus far it resembles clone A3.1; however, the former recognized only targets infected with 1934 and 1935 H1N1 viruses, while clone A3.1 recognizes all H1N1 viruses isolated between 1933 and 1943. The determinants detected by these two A virus subtype-specific clones may therefore be different.

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Table 1 Activity of T-cell clones on target cells infected with different influenza A viruses

Clone	Virus K:T	% Specific ⁵¹ Cr release			
		X31	A/Eng/42/72	A/PR/8/34	B/Hong Kong
A.31	2.5	46	4	30	1
A3.6-5.4	2.5	53	2	30	2
B8.3	2.5	46	3	26	2
B10.7	2.5	45	2	27	1
C2.2	2.5	37	3	16	1
T6-11-5-1					
Control	3	29	30	16	2

Thioglycollate-induced peritoneal exudate cells (PEC) used as targets. SR = 16–43%.

Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion

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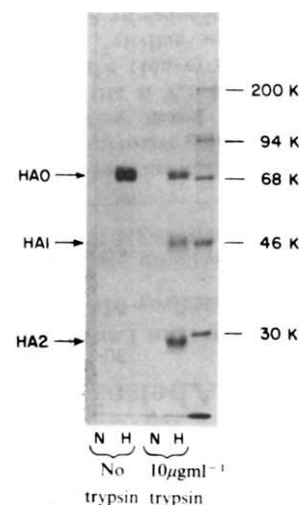
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Enveloped animal viruses enter and infect cells by a process involving fusion of the viral membrane with a cellular membrane. In some cases (for example, Sendai virus¹), the fusion event occurs at the plasma membrane; for many other viruses including influenza, entry occurs through the membranes of intracellular vesicles such as endosomes or lysosomes, where the fusion is triggered by the endogenous low pH²⁻⁶. This pH-dependent fusion activity has been studied *in vitro* using as targets cultured cells⁷⁻⁹, erythrocytes^{9,10} and liposomes¹¹⁻¹³. Fusion is a function of the viral surface glycoproteins¹² and occurs at a threshold pH that is characteristic of each virus species and strain⁸. In the case of influenza virus, there is strong evidence that the haemagglutinin glycoprotein has a key role in both virus infectivity and fusion activity^{9,12,14-16}. Both processes require a post-translational proteolytic cleavage of the haemagglutinin precursor, HA0, into the active form of the molecule, HA, which consists of two disulphide-bonded subunits, HA1 and HA2^{17,18}. A new N-terminus is generated on the HA2 subunit, the C-terminus of which is embedded in the virus membrane¹⁹. At the low pH values required for fusion the cleaved HA undergoes a conformational change exposing this previously buried hydrophobic N-terminus of HA2²⁰, possibly enabling it to interact with the target membrane. While it has been established that HA is necessary for fusion, it is unclear whether HA alone is sufficient or whether other viral proteins are involved²¹. Here we use cells expressing HA from a cloned copy of the HA gene inserted into a recombinant simian virus 40 (SV40) vector²² to demonstrate that the HA molecule displays fusion in the absence of any other influenza virus-encoded components. These results open the possibility of using HA-mediated membrane fusion as a system to deliver foreign molecules into mammalian cells.

Simian CV-1 cells infected with a recombinant SV40 virus (SVEHA3) which contains the HA gene from the Japan strain of influenza virus (A/Japan/305/57) express at the cell surface

Fig. 1 Proteolytic cleavage of HA0 on the surface of SVEHA3-infected CV-1 cells. CV-1 cells were infected with SVEHA3 as described previously²². 48 h later the cells were labelled for 1 h with ³⁵S-methionine and then washed twice with serum-free medium. Medium containing 10 µg ml⁻¹ trypsin (or lacking trypsin as control) was added to the monolayers which were then incubated for 15 min at 37 °C. Cell extracts were prepared, immunoprecipitated and analysed by SDS-polyacrylamide gel electrophoresis as previously described²². Autoradiography was for 12 h. N, normal rabbit serum; H, rabbit anti-HA serum. Molecular weight markers are indicated on the right.



large quantities of a HA glycoprotein that is indistinguishable in its antigenic, physical and biological properties from authentic influenza viral HA0²². Figure 1 shows that it is possible to cleave the HA0 into HA1 and HA2 subunits by briefly treating the cells with a low concentration of trypsin.

Monolayers of CV-1 cells were infected with SVEHA3 to obtain expression of HA0 at their surface. When treated with trypsin and briefly exposed to pH 5.0, the cells fused to form giant polykaryons (Fig. 2a). This fusion was observed from 24 h post-infection, when the cells contained ~10⁷ HA0 molecules, until just before cell lysis, which occurred at about 72 h post-infection when the cells contained ~10⁹ molecules of HA0²². No fusion occurred unless either the infected cells were exposed to low pH (Fig. 2b, d) or the trypsin treatment was omitted (Fig. 2c, d). CV-1 cells that had been mock-infected or infected with wild-type SV40 did not fuse (Table 1). Thus, the HA expressed from the SV40-HA vector if activated by cleavage into HA1 and HA2 subunits, is capable of inducing low pH-dependent membrane fusion in the absence of other influenza viral components.

The pH dependence of the cell-cell fusion in SVEHA3-infected cells is virtually identical to that observed for fusion of influenza-infected CV-1 cells or for fusion-from-without of CV-1 or BHK cells by intact Japan virus (Table 1). Thus, the membrane environment in which the HA is anchored (the plasma membrane of cultured cells or the viral membrane) has little or no effect on the pH threshold for fusion. This is consistent with the observation of Skehel *et al.*²⁰ that the conformational change in isolated HA occurs at the same pH as does activation of fusion by the intact influenza virus⁸.

Having established that the HA is necessary and sufficient for pH-dependent fusion, tests were performed to determine whether the protein had to be anchored to a membrane. The HA can be obtained, free of lipid and detergent, in three water-soluble forms: (1) as rosettes or protein micelles, in which the HA molecules are joined through their hydrophobic tails²³, (2) as free glycoprotein molecules, released from the virus membrane by digestion with bromelain (BHA)²⁴, and (3) as glycoprotein secreted from CV-1 cells infected with a recombinant vector (SVEHA20-A⁻) containing a modified HA gene that lacks the DNA sequences coding for the C-terminal hydrophobic anchor (see accompanying paper²⁵). The addition of HA rosettes or BHA spikes to monolayers of BHK cells did not cause any pH-dependent cell-cell fusion despite the fact that these cells could be completely fused from without by intact virions containing 50-fold less HA protein⁸. Furthermore, no fusion occurred when CV-1 cells infected with the anchor-minus mutant were treated with trypsin and exposed to low pH (Table 1). These results indicate that HA must be attached to a lipid matrix such as the viral envelope or the plasma membrane for fusion to take place.

Table 1 Comparison of the pH dependence of fusion of CV-1 cells by SV40-HA recombinant viruses and influenza virus

Virus	Trypsin	pH 6.0	5.5	5.3	5.1	4.9
SVEHA3	{ -	-	-	-	-	-
	{ +	-	-	+/-	+	+
SVEHA20-A ⁻ (anchor minus)	{ -	-	-	-	-	-
	{ +	-	-	-	-	-
SV40	{ -	-	-	-	-	-
	{ +	-	-	-	-	-
Mock	{ -	-	-	-	-	-
	{ +	-	-	-	-	-
Influenza (Japan)	{ -	-	-	-	-	-
	{ +	-	-	+/-	+	+
FFWO with influenza (Japan)	-	-	-	+/-	+	+

CV-1 cells were infected with the various viruses as described previously²². 24 h later the pH dependence of fusion of the cells was assayed, with or without prior treatment with trypsin, by the protocol described in Fig. 2 legend using FM adjusted to the indicated pH. The assay for fusion-from-without (FFWO) was performed as described previously⁸ using both CV-1 and BHK cells as targets. - Indicates that no fused cells were observed, + indicates that all of the cell nuclei were present in polykaryons and +/- indicates that some but not all nuclei were present in polykaryons.

It has long been known that HA is responsible for binding influenza virions to cell-surface receptors²⁶. More recently, it has become apparent that HA has a second major role in virus entry—that of fusion of the virus membrane with the membrane of endocytic vacuoles or lysosomes². We have demonstrated that a HA produced from a cloned DNA copy of the RNA gene not only expresses the binding function²², but also displays the pH-dependent fusion activity. These results show that no other influenza virus gene product is required for either of these functions. The binding and fusion activities, although carried out by the same protein, occur independently of each other. Fusion does not require sialic acid receptors in the target membrane^{11,13} and binding to such receptors does not require the cleaved form of the HA that is obligatory for fusion²². Examination of the three-dimensional structure of the HA molecule²⁷ indicates that the two functions are separated into different physical domains of the protein. The proposed receptor binding sites are located at the tips of the spikes while the hydrophobic N-terminus of HA2, which becomes exposed at low pH (ref. 20 and J. J. Skehel, unpublished) and which has been implicated as the 'fusion peptide'²⁷⁻³⁰, is found in the stem region at the base of the molecule close to where HA is anchored to the membrane.

The requirements for HA-mediated membrane fusion are (1) that the molecule be cleaved into its active form, (2) that it be anchored via its C-terminus into a lipid bilayer and (3) that it be exposed to low pH. A plausible mechanism satisfying these requirements and incorporating previous hypotheses²⁷⁻³⁰ involves the two hydrophobic regions of the protein: one of these at the C-terminus anchors the protein into the membrane of the virus (or the infected cell); the second hydrophobic peptide at the N-terminus of HA2 is exposed by the conformational change at low pH and inserts into the target membrane. This conformational change cannot occur unless the protein

has been cleaved. The HA protein now forms a bridge between the two membranes, bringing the lipid bilayers into close apposition and thus facilitating their fusion. Confirmation of this model and elucidation of the precise molecular details of the mechanism will require further studies. The feasibility of introducing mutations into the cloned copy of the HA gene, of reinserting the mutant genes into SV40-HA vectors and testing their effects on fusion activity after expression in CV-1 cells will allow definition of specific amino acids which participate in the fusion reaction.

Finally, because of its binding and fusion capacities, the HA protein may be ideally suited to facilitate the efficient delivery of foreign substances into cells. Model studies aimed at developing liposome delivery methods can be carried out using cells expressing large amounts of HA from SV40-HA vectors. Preliminary results indicate that erythrocytes can be fused into such cells.

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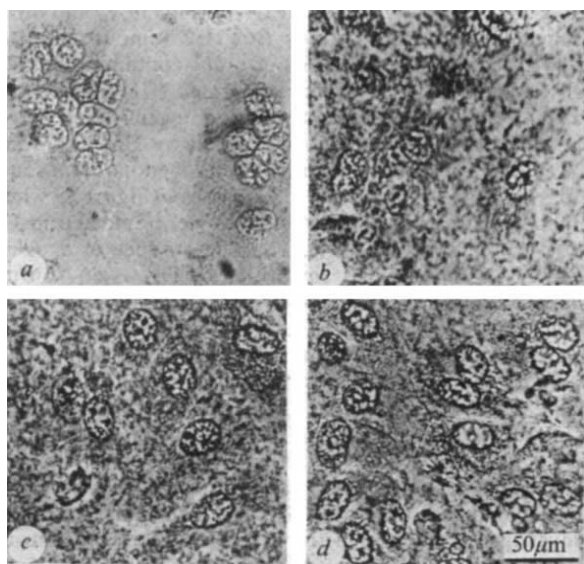


Fig. 2 Low pH-induced fusion of CV-1 cells expressing the HA of influenza virus. Confluent monolayers of CV-1 cells were infected with SVEHA3 as described previously²². 24 h post-infection, the cells were washed twice with serum-free Dulbecco's modified Eagle's medium (DMEM) and then incubated for 15 min at 37 °C in this medium (c, d) or medium supplemented with 10 μg ml⁻¹ trypsin (a, b). The cells were then washed twice with DMEM plus 10% serum and then treated for 2 min at 37 °C with fusion medium (FM) (RPMI without bicarbonate, 0.2% bovine serum albumin, 10 mM MES, 10 mM HEPES) adjusted to either pH 5 (a, c) or pH 7 (b, d). These media were then replaced with DMEM containing 2% serum and the cells placed back at 37 °C for an additional 3 h. At this time they were fixed and stained with Giemsa as described previously⁸. Photomicrographs were taken with an Olympus PM-6 camera attached to an Olympus inverted phase microscope using Pan X film.

Activation of a cellular transforming gene in tumours induced by Abelson murine leukaemia virus

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Abelson murine leukaemia virus (AbMuLV) is an acutely transforming retrovirus which induces lymphoid neoplasms in mice with latent periods of 3-5 weeks¹. The oncogenicity of AbMuLV is attributed to expression of a viral transforming gene (*v-abl*) which encodes a protein with tyrosine-specific kinase activity². Two recent findings, however, suggest that oncogenesis by AbMuLV may not be a single-step consequence of *v-abl* expression. First, Whitlock and Witte³ observed that AbMuLV infection of bone marrow cells *in vitro* stimulated proliferation of immature blast cells which did not exhibit the *in vitro* growth properties or tumorigenicity characteristic of cells from AbMuLV-induced neoplasms. These AbMuLV-infected cell populations evolved to fully neoplastic cells only

after several weeks in culture, suggesting that secondary events were required for expression of the neoplastic phenotype³. Second, Grunwald *et al.*⁴ reported the loss of the AbMuLV genome after prolonged *in vivo* passage of some AbMuLV-induced neoplasms, suggesting that expression of *v-abl* may be necessary for initiation, but not maintenance, of transformation. We report here that AbMuLV-induced neoplasms contain cellular transforming genes detectable by transfection which are distinct from AbMuLV sequences. Taken together, these results suggest that *v-abl* expression may induce early events in the neoplastic process and that secondary activation of a distinct cellular transforming gene may be involved in progression to neoplasia.

DNAs used as donors in transfection assays were extracted from two cell lines, B6T1E2 and B6T4E4, derived from early (fifth) *in vivo* passages of neoplasms induced by injection of newborn C57BL/6 mice with AbMuLV⁴. These tumour cell lines contain exogenously integrated AbMuLV DNA, express the *v-abl*-encoded transforming protein p160, and exhibit phenotypic markers (Thy-1-negative, Lyt-1-negative, Lyt-2 negative, immunoglobulin-negative, DNL1.9-positive⁵ and 14.8-positive⁶) characteristic of AbMuLV-induced pre-B-lymphocytic neoplasms (ref. 4 and R. Risser, personal communication).

B6T1E2 and B6T4E4 DNAs induced transformation of NIH 3T3 cells with high efficiencies (approximately 1 transformant per μg DNA; Table 1). These transforming efficiencies are comparable to those obtained with DNAs of other human or mouse lymphoid neoplasms⁷, but are at least 200-fold higher than the transforming efficiency of normal cell DNAs. Several foci of NIH cells transformed by each tumour DNA were grown to mass culture for further study. DNAs of these transformed NIH cells induced transformation in secondary transfection assays with efficiencies comparable to those obtained with the primary tumour DNAs (see Table 1). These results indicate that AbMuLV-induced neoplasms contain activated transforming genes which can be detected and serially passaged by transfection of NIH 3T3 cells.

The content of AbMuLV DNA in NIH cells transformed by these tumour DNAs was investigated by restriction endonuclease digestion and blot hybridization using a *v-abl*-specific fragment of a cloned AbMuLV provirus (λ AM-1)⁸ as probe. *SacI* digestion of normal mouse DNA yielded three endogenous *c-abl*-containing fragments with molecular weights of 16, 5.8

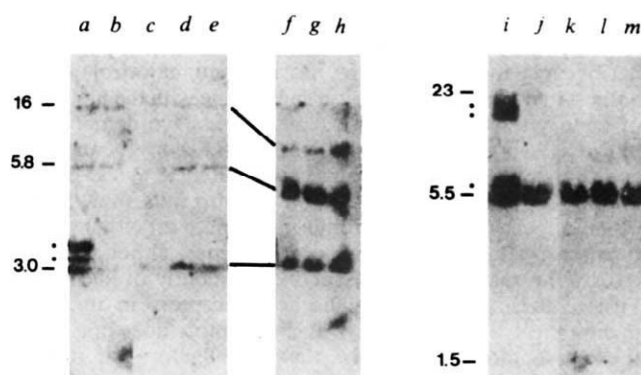


Fig. 1 Analysis of *abl* sequences in transformed NIH cells. Cellular DNAs were digested with either *SacI* (a-h) or *BamHI* (i-m), electrophoresed in agarose gels and analysed by Southern blot hybridization¹⁸ as described previously⁷ with ³²P-DNA probe prepared by nick-translation of a 1.5-kb *v-abl*-containing *Bgl*III fragment of λ AM-1 (ref. 8). a, i, B6T4E4 DNA; b, j, NIH 3T3 DNA; c-e, k-m, DNAs of three independent lines of NIH cells transformed by B6T4E4 DNA; f-h, DNAs of three independent lines of NIH cells transformed by B6T1E2 DNA. The molecular weights of the endogenous normal mouse *c-abl* fragments⁹ are indicated in kb. Dots indicate exogenous *v-abl* fragments in B6T4E4 DNA.

and 3.0 kilobase (kb)⁹ (Fig. 1). As *SacI* cleaves within the AbMuLV long terminal repeat present at both ends of integrated viral DNA and at one internal site in the *v-abl* sequences⁹, DNA of AbMuLV-infected cells (for example, B6T4E4 tumour cells) yielded two additional exogenous *v-abl*-containing *SacI* fragments of 3.2 and 3.5 kb (Fig. 1a). Similar exogenous *v-abl* *SacI* fragments were detected in B6T1E2 DNA (not shown). In contrast, DNAs of three NIH cell lines transformed by B6T4E4 tumour DNA (Fig. 1c-e) and of three NIH cell lines transformed by B6T1E2 tumour DNA (Fig. 1f-h) yielded only the endogenous *c-abl*-containing *SacI* fragments detected in DNA of untransformed NIH 3T3 cells (Fig. 1b). These observations were confirmed by analysis of *BamHI*-digested DNAs. NIH cells transformed by B6T4E4 tumour DNA (Fig. 1k-m) contained only the endogenous *c-abl* sequences present in DNA of NIH 3T3 cells⁹ (Fig. 1j), whereas additional exogenous *v-abl* sequences were detected in DNA of B6T4E4 tumour cells (Fig. 1i). The absence of exogenous *v-abl* DNA in NIH cells transformed by DNAs of AbMuLV-induced tumours indicates that the transforming activity of these tumour DNAs is not mediated by transfer of the AbMuLV genome. As none of the six transformed NIH cell lines contained exogenous AbMuLV sequences, it seems that the transforming activity of AbMuLV DNA is significantly lower than that of the transforming gene detected in these transfection assays.

The transforming genes detected by transfection of AbMuLV-induced tumour DNAs were further characterized by investigating their sensitivity to cleavage with restriction endonucleases. Transforming activities of DNAs of both tumours studied were inactivated by digestion with either *BamHI* or *XhoI* but not by digestion with *EcoRI* or *HindIII* (Table 2). In contrast, transforming activity of AbMuLV DNA is not affected by digestion with *BamHI*¹⁰. The susceptibility of AbMuLV-induced tumour DNAs to restriction endonuclease digestion is the same as that previously reported for DNAs of four human pre-B-lymphocytic neoplasms, but differs from the patterns of inactivation observed for transforming activities of DNAs of human and mouse neoplasms representing intermediate and mature stages of B- and T-lymphocyte differentiation⁷. These results indicate that the transforming gene detected by transfection of DNAs of AbMuLV-induced mouse pre-B-lymphocyte neoplasms is closely related to the transforming gene activated in human pre-B-lymphocyte neoplasms.

Table 1 Transforming activity of DNAs

Donor DNA	Total foci/total recipient cultures	Foci per μg DNA
<i>AbMuLV-induced tumours:</i>		
B6T4E4	321/14	1.1
B6T1E2	416/16	1.3
<i>Transformed NIH cells:</i>		
NIH (B6T4E4) 3-2	51/6	0.43
NIH (B6T4E4) 3-1	85/6	0.71
NIH (B6T4E4) 1-1	93/5	0.93
NIH (B6T1E2) 4-1	56/6	0.47
NIH (B6T1E2) 1-2	58/6	0.48
NIH (B6T1E2) 1-1	47/6	0.39
<i>Controls:</i>		
Normal mouse thymocytes	0/15	<0.003
Salmon sperm	6/100	0.003
Normal mouse liver	0/20	<0.003
NIH 3T3	0/25	<0.002

NIH 3T3 cells were exposed to 20 μg of donor DNA per recipient cell culture and foci of transformed cells were counted 11-13 days after transfection⁷. B6T4E4 and B6T1E2 are AbMuLV-induced mouse pre-B-lymphocyte neoplasm cell lines. Three independent foci of NIH cells transformed by each of these tumour DNAs (designated NIH (B6T4E4) and NIH (B6T1E2) cells) were grown to mass culture and used as donors of DNA in secondary transfection assays. Control DNAs were from normal mouse tissues, NIH 3T3 cells and salmon sperm.

Table 2 Digestion of tumour DNAs with restriction endonucleases

	Undigested	Foci per μg DNA			
		<i>Bam</i> HI	<i>Eco</i> RI	<i>Hind</i> III	<i>Xho</i> I
B6T4E4	1.0	0.01	1.1	1.2	0.06
B6T1E2	0.75	0.01	0.95	1.3	0.03

DNA (80 μg) of AbMuLV-induced tumours was digested to completion with the restriction endonucleases indicated. Bacteriophage λ DNA (2 μg) was included in a separate aliquot of each reaction that was incubated in parallel and analysed by electrophoresis in agarose gels to monitor the specificity and extent of digestion. Transforming activities of digested and undigested DNAs were assayed by focus formation on NIH 3T3 cells.

Previous studies have identified transforming genes in neoplasms induced by the weakly oncogenic retroviruses avian lymphoid leukaemia virus (LLV), mouse mammary tumour virus (MMTV) and murine leukaemia virus (MuLV)^{7,11,12}. In contrast to acute transforming retroviruses such as AbMuLV, these weakly oncogenic retroviruses do not contain specific viral transforming genes and require long latent periods (>6 months) for induction of neoplasia. The transforming genes detected by transfection of DNAs of LLV-induced chicken B-cell lymphomas and of MMTV-induced mouse mammary carcinomas were not linked to viral DNA sequences, indicating that oncogenesis by these viruses involves indirect activation of cellular transforming genes^{11,12}. In addition, restriction endonuclease analysis indicated that the transforming genes activated in MMTV-induced mammary carcinomas, or in MuLV-induced lymphoid neoplasms, were also activated in chemically induced or spontaneously occurring neoplasms of the same cell types^{7,12}.

Induction of chicken B-cell lymphomas by LLV also involves activation of a cellular gene (*c-myc*) homologous to the transforming gene of the acute transforming virus MC-29, by adjacent integration of exogenous LLV transcriptional regulatory sequences¹³. However, the transforming gene detected by transfection of chicken B-cell lymphoma DNAs is not homologous to *c-myc*, indicating that LLV-induced B-cell lymphomas contain at least two distinct transforming genes¹⁴. Since induction of lymphomas by LLV seems to be a multi-step process, it has been suggested that the two distinct transforming genes may act at different stages of neoplastic development¹⁴.

The present experiments suggest that oncogenesis by an acute transforming virus, AbMuLV, can also involve indirect activation of a cellular transforming gene. As only two tumours were analysed in these experiments, further studies will be required to assess the generality of the results. Together with the recent findings of Whitlock and Witte³ and Grunwald *et al.*⁴, however, our results suggest the possibility that the *v-abl* gene product acts at an early stage of oncogenesis and that progression to neoplasia involves activation of a distinct transforming gene detected by transfection. Rather than inducing transformation as a single-step event, the role of *v-abl* in the neoplastic process may be similar to that of the activated *c-myc* gene in LLV-induced chicken lymphomas.

Restriction endonuclease analysis of the transforming gene detected by transfection of DNA of AbMuLV-induced pre-B-lymphocyte neoplasms indicates that this gene is closely related to one previously detected in human neoplasms of the same cell type⁷. AbMuLV has also been reported to induce neoplasms of more mature B lymphocytes¹⁵, T lymphocytes¹⁶ and myeloid cells¹⁷. It will be of interest to characterize cellular transforming genes activated in AbMuLV-induced tumours of these other cell types in relation to the stage-specific transforming genes reported in other mouse and human B- and T-lymphocyte neoplasms⁷.

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Cooperative deoxygenation of haemoglobin: asymmetry of binding and subunit differences

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Oxygen uptake by the blood in the lungs of a resting human brings the level of saturation of haemoglobin from ~70% to >97% (refs 1, 2). The recourse to this upper portion of the oxygen binding curve is reflected in an asymmetry of the isotherm which is frequently reported^{3,4}, but which has not been accorded any physiological role. This asymmetry is responsible for a more pronounced cooperativity of the deoxygenation at nearly complete saturation than that displayed in the typical sigmoidal pattern of loading at low oxygen tensions. Such behaviour is viewed here as a consequence of and a possible rationalization for the presence of two similar but distinct types of subunit in the haemoglobin tetramer.

A binding process would be defined as symmetrical if the fractional extent of saturation, θ minus $1/2$, plotted against $\log(P/P_{1/2})$, where P is the oxygen tension and $P_{1/2}$ that corresponding to $\theta = 1/2$, yielded a curve with inversion symmetry about the origin. For ligation to a tetramer this symmetry condition would require⁵ that $\sigma \equiv (K_{2d}K_{3d})/(K_{1d})(K_{4d}) = 1$ where K_{1d} – K_{4d} are the intrinsic dissociation constants for the first to the fourth attached oxygen, respectively.

The first and last dissociation constants (K_{1d} and K_{4d}) are obtainable by extrapolation³ as indicated in Fig. 1a,b. Greater uncertainty applies to the determination of K_{2d} and K_{3d} but their more reliably determined product⁶ is sufficient, with K_{1d} and K_{4d} , to specify σ (designated W previously)^{3,4}. Deviation of this parameter from unity has been adduced as evidence against several models which seek to express the four dissociation constants in terms of a smaller number of parameters^{5,7,8}.

From extant data for human haemoglobin stripped of diphosphoglycerate (DPG), a value of $\sigma = 29$ can be calculated⁹, while $\sigma = 47$ for the same haemoglobin in the presence of DPG¹⁰. In contrast, a value of 84 has been reported for whole human blood⁴. Thus there is considerable variability in this parameter, due to the uncertainties in K_{2d} and K_{3d} , and because of differences in solution conditions and in measurement techniques. No values of $\sigma < 1$ have been reported and the phosphorylated co-factors^{11,12}, when present, elevate this parameter.

The relationship of σ to the pattern of oxygen ligation can be seen by comparing the rates of change of the slopes in Fig. 1. For the sigmoidal region at low oxygen tensions in Fig. 1a

$$[d^2\theta/d(P/K_{1d})^2]_{P=0} = 2[3(K_{1d}/K_{2d}) - 4] \quad (1)$$

while the comparable quantity for the dissociation curve in Fig. 1b is given by

$$[d^2(1-\theta)/d(K_{4d}/P)^2]_{P\rightarrow\infty} = 2[3(K_{3d}/K_{4d}) - 4] \quad (2)$$

The introduction of the factors K_{1d}^2 and K_{4d}^2 renders these derivatives independent of the choice of the standard state for the ligand. For a hyperbolic binding curve, as found with myoglobin or with independent binding and four identical intrinsic constants, both derivatives equal -2 .

The ratio of the second derivative in equation (2) to that in equation (1) is greater than or equal to σ . It is equal only if $\sigma = 1$, in which case, changes of abscissae in the two graphs to P/K_{1d} and K_{4d}/P would make the curves superposable—another consequence of symmetry. With the cited values of σ , the sigmoidality of the dissociation curve in Fig. 1b as characterized by the second derivative can be 30–100 times greater than that shown in the customary binding curve of Fig. 1a. The asymptotic flattening in the latter provides no visual hint of the marked cooperativity of deoxygenation. Haemoglobin loads oxygen at a pH higher than it yields it up (Bohr effect), as indicated in Fig. 1a. The ratio of the sigmoidality parameters might be anticipated to be even greater at pH values lower than the slightly alkaline ones for which the σ s quoted here apply.

A search for the origin of this asymmetry focuses naturally on the pairs of α - and β -subunits comprising the haemoglobin tetramer. A model can be advanced along the lines of those proposed previously⁷ which allows for variations in reaction with oxygen of these two types of polypeptide chain in the tetramer. Such a model must minimally contain two intrinsic dissociation constants $K_{\alpha d}$ and $K_{\beta d}$ for the desorption of the first oxygen bound, respectively, to one α - and one β -subunit. Four interaction parameters, $\rho_{\alpha\alpha}$ and so on (see Fig. 2), measure the extent of diminution of dissociation from one haem site effected by oxygens occupying neighbouring sites. These quantities are expected to have an upper limit of unity with strong repression of dissociation characterized by magnitudes substantially less than one. They can be related directly to the conditional free energies of site occupancy¹³. Expressions for the four intrinsic dissociation constants involve all six of the above parameters. The asymmetry is given by

$$\sigma = [(K_{\alpha d} + K_{\beta d})^2 \rho_{\alpha\alpha} \rho_{\beta\beta}] / [(K_{\alpha d} \rho_{\alpha\alpha}) + (K_{\beta d} \rho_{\beta\beta})]^2 \quad (3)$$

The cross-interaction parameters $\rho_{\alpha\beta}$ and $\rho'_{\alpha\beta}$, of which two were introduced because each subunit is differently disposed structurally in the tetramer with respect to its two dissimilar

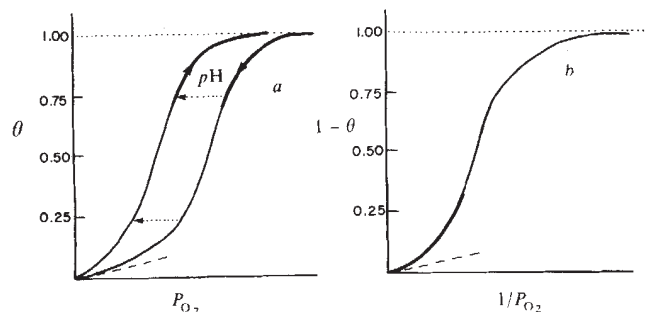


Fig. 1 a, Customary curves for oxygen loading (left) and unloading (right) by haemoglobin. The darker portions of the curves indicate the operative region in resting humans. The broken line is the initial tangent given by $[d\theta/dP]_{P=0} = 1/K_{1d}$. b, Fractional extent of unsaturation plotted against $1/P$ to illustrate the highly cooperative deoxygenation reaction. The limiting tangent at high oxygen tension as shown by the dashed line is $[d(1-\theta)/d(1/P)]_{P\rightarrow\infty} = K_{4d}$.

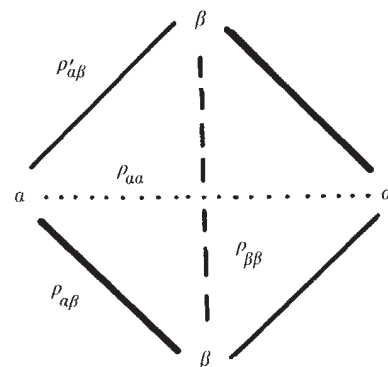


Fig. 2 The haemoglobin tetramer with six edges marked by parameters for repression of dissociation induced by intersubunit contacts. They are formally represented as two site-site interactions between like neighbours ($\alpha\alpha$ and $\beta\beta$) and two ($\alpha\beta$ and $\alpha\beta'$) pairs of interactions between unlike partners.

neighbours¹⁴, are absent from equation (3). This is because their magnitudes, which affect equally both α and β , cannot contribute to the asymmetry. From equation (3) $\sigma = 1$ if $\rho_{\alpha\alpha} = \rho_{\beta\beta}$ or $K_{\alpha d} \rho_{\alpha\alpha}^2 = K_{\beta d} \rho_{\beta\beta}^2$.

The condition of practical interest of $\sigma > 1$ can be shown to mandate either of two pairs of inequalities, that is, $\rho_{\alpha\alpha}/\rho_{\beta\beta} > \sigma \approx 30$ –90 and $K_{\beta d}/K_{\alpha d} > \sigma^{1/2} \approx 5$ –9, or the opposite obtained by interchanging α and β . Site-site as opposed to single site interaction accounts for the higher power of σ appearing in the first inequality. This is the experimentally favoured pair as it requires that the β -chains, which self-aggregate and are in more intimate contact¹⁴ in haemoglobin A, are the more efficacious in depressing the dissociation constants. Also, the companion inequality is consistent with reports^{15–17} that the α -chains are the first to bind oxygen.

While intense physical activity can cause blood in the pulmonary capillary bed to be more deprived of oxygen¹ than the 70% level cited above, the usual oxygen tension must convert it overwhelmingly (and rather rapidly¹⁸) to quite high extents of saturation. Thus the cooperative character to the oxygenation must be of secondary importance to that exhibited by the deoxygenation in states of physiological stress. The asymmetry of the ligation clearly serves this purpose. An asymmetry of reaction assignable to a structural cause is comprehensible. However, the existence of two kinds of subunit in haemoglobin is of wide occurrence, even among primitive vertebrates. Is its origin as well as its preservation attributable to the need for these diverse organisms to maintain a capacity for an asymmetric response to oxygen requirements?

Many of the conclusions about the quantitative behaviour of haemoglobin have been reached independently by Dr Gregorio Weber through consideration of the general consequences of asymmetric ligation. I thank Drs John Severinghaus and Peter Torgerson for helpful comments and the NSF for support (grant PCM-8022006).

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BOOK REVIEWS

A philosopher among the physicists

Donald MacKay

SIR Karl Popper is not the kind of original thinker who is content to swim peacefully against the stream of contemporary fashion. Instead, he takes Puckish delight in splashing the water in the faces of those who drift unthinkingly by. "Many younger physicists", he laments, "have grown up in a period of over-specialisation, and in the newly developing tradition of the cult of narrowness, and the contempt for the non-specialist older generation . . . a tradition which may easily lead to the end of science and its replacement by technology". He sees the current scientific orthodoxy as riddled with philosophical errors and confusions that urgently need sorting out; but where a lesser mind might be moved to attack these with grim and tedious analyses in abstract terms, it is Popper's genius to make the issues come alive in terms of actual events and concrete problems in the history of science, as seen by the people who faced them. The result is extraordinarily stimulating, whether or not one agrees with him. To come to grips with his thinking is to have one's mental muscles enjoyably stretched, and one's presuppositions submitted to searching scrutiny in an almost playfully challenging — though deadly serious — spirit.

These two volumes are part of a trilogy based on Popper's legendary *Postscript to The Logic of Scientific Discovery*, which after circulating privately for 25 years has now acquired a variety of qualifying and amplifying addenda. In Vol. II, *The Open Universe*, Popper is concerned mainly with refuting the idea that the world is deterministic in any of several senses which he explores. Three addenda argue for the reality of worlds other than the physical, and against ontological reductionism. Volume III, *Quantum Theory and the Schism in Physics*, is a hard-hitting attack on the subjectivist interpretation of quantum mechanics favoured by the Copenhagen School, and on what Popper sees as kindred interpretations of the Second Law of Thermodynamics. It includes a detailed argument for his well-known explication* of probability as "objective propensity", and ends with a "Metaphysical Epilogue" on the nature of science and its open problems, as seen in terms of his objectivist propensity interpretation.

What invigorates in Popper is his robust

Postscript to the Logic of Scientific Discovery, Vols II and III. By Karl Popper, edited by W.W. Bartley, III. Vol. II *The Open Universe: An Argument for Indeterminism*. Pp.185. ISBN 0-09-146180-4. Vol. III *Quantum Theory and the Schism in Physics*. Pp. 229. ISBN 0-09-146170-7. (Hutchinson/Rowman & Littlefield: 1982.) Each volume £15, \$24.50. Vol. I, *Realism and the Aim of Science*, has just been published in the United States and will appear in Britain early next year.

and commonsensical realism. "Realism", he declares, "is linked with rationalism, with the reality of the human mind, of human creativity, and of human suffering". He scorns claims such as Wigner's that the laws of quantum mechanics cannot be formulated without recourse to the concept of consciousness, as

based on bad philosophy and on a few very simple mistakes [which] I hope will soon be forgotten, while the great physicists who happened to commit them will be forever remembered by their marvellous contributions to physics: contributions of a significance and depth to which no philosopher can aspire.

He rebuts the idea that von Neumann conclusively proved the impossibility of "hidden variables"; and he effectively demolishes much subjectivist nonsense talked about the famous "Reduction of the wave packet". No teacher of quantum theory should miss the opportunity to sharpen his thinking against this abrasive whetstone, especially if he wants his pupils to distinguish solid science from fashionable dogma.

In his Epilogue Popper strikes an optimistic note, suggesting that by replacing talk of "particles" by talk of "propensities" as the objectively real elements of the physical theory of matter, the wave-particle dualism of orthodox theory could be dispensed with, and the dispute between Einstein's and Born's interpretations resolved. Particles, he tells us, "can be produced by propensities, and . . . up to a point . . . are propensities" (Vol. III, p. 196). One might have expected propensities to be linked more closely with events than with entities — with particle-impact and the like, rather than with particles; but Popper evidently has in mind Dirac's theory of the positron as an analogous line of thought (Vol. III, p.197). The Epilogue unfortunately does not go into enough detail to allow the fruitfulness of his suggestion to be assessed.

In his hot pursuit of subjectivity, there are times when Popper's polemical zeal drives him to cut some corners. Against subjectivist notions of entropy, for example, he offers as an objective criterion of disorder in a system the "chancelikeness" of the distribution of its molecules (Vol. III, p.112). Obvious counter-examples spring to mind. If a (highly-ordered) busload of soldiers scatter to precisely specified but randomly distributed hiding places, are they now any less orderly? Is the sequence of digits down the columns of a telephone directory "disorderly"? Compactness is only one form of order; and dispersion is not the same concept as disorder. Everything here depends on the data that determine the distribution of the dispersed members. To claim that Boltzmann's entropy-measure is *relative* to a specific data-base does not make it subjective; and the link between disorder and lack of information cannot be dismissed quite as easily as Popper suggests.

A passionate concern for human freedom and dignity shines through Popper's polemics against determinism, which he sees as incompatible with responsible choice and human creativity. He argues convincingly that deterministic concepts of our world have no scientific support; and he sets out in improved form his famous 1950 proof that no discrete-state computing machine could predict precisely the future of a universe that included itself, even in terms of the laws of classical (pre-Heisenberg) physics.

Even if we accept all this, however, and thoroughly sympathize with its motivation, some nagging doubts remain. First, as Popper himself acknowledges, our inability to predict a future event is neither a necessary nor a sufficient condition of its being undetermined; so his heavy emphasis on the unpredictability-to-us of the future can here be misleading and even counterproductive. Secondly, indeterminism would be of value in this connection only if it allowed scope for the control of human actions by human conscious choices; but for this Popper can only ask us to picture the mind (in a non-physical "World 2") as "interacting" with the brain (in "World 1"), without offering any scientific account of how such interaction could take place, let alone how his conjecture could be falsified. (His more extensive treatment of the topic with Sir John Eccles in *The Self and its Brain* (Springer-Verlag, 1977) still leaves these matters unclear.)

*For Popper the "probability" of a physical event denotes an objective physical property of the generating situation, which he terms its "propensity" to produce the event.

My main doubt, however, is whether Popper has shown human freedom and creativity to *require* the brain to be physically indeterminate in its workings. He rejects — for good reasons — the simple equation of mind with brain; but he ignores another option which, as I see it, would safeguard all the values he wants to maintain without the awkward implications of interactionism. This would

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Karl Popper — “extraordinarily stimulating, whether or not one agrees with him”.

see mental activity, rather than *interacting with* the brain, as *embodied in* brain activity, in something of the sense in which an equation being solved by a computer is embodied in the computer's physical structure at the time. To say that the behaviour of a computer is determined by the equation it is solving, is quite compatible with saying that its physical workings are determined by the laws of physics. Indeed, from the programmer's standpoint, the latter may be a necessary condition of his claim; and there is certainly no need to prove the computer open to “non-physical influences from another world” in order to sustain that claim.

Scientific determinism (in Popper's sense) may well be false and baseless; but for his elaborate attack on it to be of much consequence for human freedom he needs to show why the physical determinateness of brain processes would preclude the capacity of human beings to determine their actions freely in accordance with their conscious thinking, evaluating and choosing. As I have argued elsewhere — see *Mind* 69, 31–40 (1960), and *Human Science and Human Dignity* (Hodder and Stoughton, 1979) — even if the outcome of such choosing were predictable by non-participant observers, it would not

logically follow that the outcome was inevitable for the chooser himself, nor that *he* had really no options open to him; for this, too, is an objectively relativistic situation. What the non-participants would be correct to believe about the immediate future of the chooser's cognitive mechanism is something the chooser himself would in general be mistaken to accept as accurate and inevitable-for-him, since the physical changes required for its embodiment in his brain cannot but significantly affect its accuracy. Thus even the complete success of a prediction kept secret from the chooser until after the event would not show in retrospect that *he* had no other option open to him; for no prediction of the outcome existed, even unknown to him, that had an unconditional claim to *his* assent. There is, after all, nothing particularly shocking in the thought that the form of a future event may be fixed-and-inevitable relative to someone not causally coupled to it, while still indeterminate and open to the individual whose thinking and deciding will constitute the event in question.

This relativistic point bears particularly on Popper's dispute with Einstein over the implications of the latter's deterministic world-model, as narrated in Vol. II, pp. 89–92. According to Popper, Einstein's views in 1950 implied that the future is contained in the past “as the chick in its egg”. This, Popper argued, made the future “superfluous”, and our sense of time “illusory”. Moreover, “if we were experiencing successive [motion picture] shots of an unchanging world, then one thing at least was genuinely changing . . . our conscious experience” (Vol. II, p. 92), and this “looked very much like a flat contradiction”. As soon as we distinguish the different standpoints here, however, all appearance of contradiction vanishes.

Courtesy Hutchinson

From the standpoint of the characters in a film (or a history book) their future is asymmetrical with their past in that, willy nilly, what they think and desire and do will play a part in determining its form. Their experience of change is not an illusion, but a fact of their world that they would be lying to deny. From the standpoint of the film-projectionist (or the book-reader), of course, the future of the characters is as much a fixed and unchanging fact-for-him as their past. It is “all in the book”. To regard these views as logically conflicting, however, is surely a mistake; for the data available to the projectionist (or the bookreader), so far from showing the experience of the characters to be “illusory”, actually supply confirmatory evidence of the changes experienced in their world, and of the asymmetry-to-them of past and future. It is thus rather touching to be told that the ageing Einstein, when confronted with Popper's arguments, merely “said that he was impressed by them, and that he had no answer for them” (p. 92).

A reviewer's task is to be critical where he must; and indeed only lack of space precludes comment on a number of other matters, such as Popper's interpretation of the quantum-mechanical ψ -function, his tendency to confound rationality and rationalism, and his inclination to take “World I” (the world of physical objects) as the “standard example of reality”. Let me conclude, however, by warmly recommending the catalytic experience of reading — and re-reading — these mature and scholarly reflections of a great mind, on matters of central concern to physical science and of importance to us all. □

Donald MacKay, a physicist, is Emeritus Professor of Communication and Neuroscience at Keele University.

Digging deep into plasma physics

T.J.M. Boyd

The Interaction of Strong Electromagnetic Fields with Plasmas. By I.R. Gekker. Pp. 324. ISBN 0-19-851467-0. (Oxford University Press: 1982.) £35, \$85.

THE coming of age of plasma physics has been reflected in its literature by a steady growth in the output of monographs as compared with the primers of years past. There has appeared lately a stream of more or less specialized works addressing topical — occasionally, by the time the books appear, not-so-topical — aspects of what is now a wide-ranging subject. Professor Gekker's book on the interaction of strong electromagnetic radiation with plasmas is avowedly specialist, and its content undeniably topical.

The text is in two parts. Part I

summarizes the simple linear physics of plasma wave propagation, with a short excursion into electromagnetic wave absorption in inhomogeneous plasmas which serves as an introduction to the theme of the work proper. Part II consists of six chapters devoted to aspects of the nonlinear physics of propagation and absorption of electromagnetic waves, a subject to which Professor Gekker has himself contributed widely. Starting with a statement of the properties of nonlinear phenomena in plasmas — at four pages a *précis* of almost unbecoming brevity — Part II goes on to develop the interaction physics of charged particles with high frequency fields in both magnetized and unmagnetized plasmas. This is followed by a chapter on the interaction of strong

electromagnetic fields with dense plasmas, altogether too ambitious a title in view of its very restricted compass. The final two chapters on the anomalous absorption of electromagnetic radiation and concomitant plasma heating are, by contrast, full and detailed and make up almost half the text. The book concludes with an extensive bibliography and a less than comprehensive index.

The work is given a distinctive flavour by its emphasis not only on the experimental aspects of the interaction of radiation with plasmas but on details of the experimental procedures themselves. While there are undeniably circumstances in which this amount of detail is valuable — even essential — one is left with a clear impression that on many occasions the descriptions provided serve no purpose and could have been dispensed with to advantage. Worse, however, is that they sometimes positively get in the way of an otherwise informative discussion. Examples of such over-indulgence are to be found in virtually every section of Part II. Some read like paraphrases of the original literature; others are probably relics from the lectures on which the book is based and would have been best left out. Had Professor Gekker been less prodigal in his attention to descriptive detail he might have felt able to devote correspondingly more space to discussing the implications of the various experiments for nonlinear plasma physics.

Another shortcoming, perhaps not unrelated to the book's origins in a course of lectures, is some tendency to be comprehensive rather than critical. While it is informative to have one experiment compared and contrasted with another, there is perhaps less merit in setting out a catalogue of work done without critical commentary. Another aspect of the same problem manifests itself in the lists of authors which are interspersed in the text, extending in one instance to 12 lines of unnecessary interruption. More substantial than these pinpricks is the considerable imbalance in the treatment of the subject matter. For example Chapter 9 forms almost one-third of the text, with one section alone (Section 9.5 on microwave absorption experiments) extending to 35 pages. This could have been subdivided to advantage.

These various criticisms should not be taken to imply that the book is without merit. It is a veritable mine of information — albeit one in which much of the ore is deep level — and will repay careful reading. The readership is likely to consist in large part of scientists active in this area of plasma physics. Unlike the original Russian version this translation is less likely to be used as a text in postgraduate courses though it could, and should, find a place on reading lists. □

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Abscission since Newton

Roy Sexton

Abscission. By Fredrick T. Addicott with illustrations by Alice B. Addicott. Pp.369. ISBN 0-520-04288-3. (University of California Press: 1982.) £29.75, \$51.35.

LIKE MOST abscission physiologists I am occasionally confronted by some hearty soul who tells me that we would probably know more about abscission if only Newton had confined his thoughts to the processes that occurred just prior to the fall of the apple. The implication that there are major gaps in our knowledge of abscission is well founded but unfortunately these problems have failed to attract the attention they merit from botanists.

In the preface to *Abscission*, F.T. Addicott makes plain his aim to interest plant scientists from a wide range of backgrounds. Unlike most previous reviewers, he has not confined his attention to the physiology and agronomic importance of the process but has expanded the scope to include the lower plants and topics such as the genetics and ecology of abscission. During his 40-year association with the subject the author has accumulated 2,500 papers concerned with abscission and as a result he is able to write with unrivalled authority on most aspects of the process. The obvious zeal with which this information has been gathered is impressive, though great care has been taken not to overwhelm the reader and the information is presented in a concise yet readable form. There is little doubt that the massive effort involved in mastering this material has been worthwhile and will ensure that this volume will become the standard reference work for many years to come.

The first section of the book describes the various structures that can be shed from the plant, ranging in size from the smallest trichomes to the complete aerial portion of tumble weeds. Detailed accounts are given of the anatomy, cell biology and biochemistry of the weakening process. The section on the hormonal control of abscission is briefer than I had anticipated, and although the literature coverage is adequate it is not as comprehensive as that in other chapters. This section contains a spirited defence of the view that abscisic acid is a major controlling factor in abscission, which is perhaps what one would expect from a member of the team involved in the original characterization of the substance.

Also included are a chapter describing the increasing agronomic importance of manipulating abscission and another concerned with the ecological significance of different abscission strategies. The sections on the genetics and evolution of abscission are especially noteworthy because they represent the first serious attempts to review these subjects.

The front cover of the book carries not only the author's name but also that of his wife, Alice B. Addicott, who was responsible for the fine botanical drawings. This pleasant feature draws attention to the unrecognized yet important role that illustrators play in improving the clarity of scientific texts. However I appreciate mention of her name for another reason. I was once asked by a student why the recognized abbreviation for abscisic acid was ABA (see *Science* **159**, 1493; 1968) and not AbA which seemed more logical. Is this perhaps just a coincidence; or is there another explanation? □

Roy Sexton is a Lecturer in the Department of Biological Science at the University of Stirling.

Variety in singular behaviour

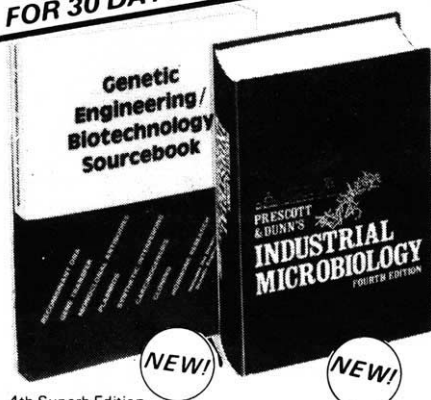
N.B. Davies

The Shelduck: A Study in Behavioural Ecology. By I.J. Patterson. Pp.276. ISBN 0-521-24646-6. (Cambridge University Press: 1982.) £27.50.

THE CURRENT trend for research in behavioural ecology is first to think of an interesting idea and then of a species that will be good for testing it. Detailed studies of all the various aspects of a single species have become less fashionable and are sometimes disparagingly labelled "the aardvark approach"; if no one has yet studied aardvarks then we use this as the main excuse for doing research on them. This view is unfortunate because we have only to think of Lack's robins, Nice's sparrows and Tinbergen's gulls to remind us that some of the main ideas in behaviour and ecology have been derived from long-term studies of particular species.

Shelducks have been studied on the River Ythan in Scotland for the past 20 years by Ian Patterson and students from Aberdeen University, forming the subject of six theses and over 20 published papers. This book, which summarizes the work and relates it to other studies, is a good example both of how general theories can best be tested with long-term data and also of how a detailed study of one species can give rise to new ideas of wider interest. The book is organized around the shelduck's annual cycle, beginning with the extraordinary autumn migration to the mudflats of north-west Europe where, safe from predators, thousands of birds congregate to moult, dropping all their flight feathers

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simultaneously and so becoming flightless for about a month. Then there follow chapters on winter flocking, territories, nest sites, laying and incubation, care of the young and population regulation.

The first workers in the 1960s interpreted much of the shelduck's social behaviour and population dynamics in relation to Wynne-Edwards's theory of reproductive restraint by individuals in order to prevent over-population. The groups of birds which assembled in the sand dunes in spring were regarded as "parliaments", where decisions were made for the good of the group as to who should breed and who should be excluded. Patterson's studies have shown that this behaviour makes much better sense from a selfish-individual point of view. The non-breeders turn out to be mainly young birds prospecting for nest burrows late in the season, probably to gain familiarity with the breeding area for the next year. The parliaments appear to involve subordinate or previously unsuccessful birds following dominants around to parasitize their knowledge of nest sites. There is also the possibility that "non-breeders" in fact dump eggs in the breeders' nests; up to half of the nests in some years contain eggs laid by more than one female. Shelducks are so shy, however, and their nests at the ends of rabbit burrows are so inaccessible, that the details of these multiple clutches are still unknown.

Equally fascinating is the phenomenon of brood mixing. When parents escort their ducklings from their burrows in the dunes to feeding grounds on the estuary, they often encounter other families. During the aggressive encounters between adults, ducklings often attach themselves to the wrong parents. Some pairs may lose all their young while others accumulate large crèches. The data on duckling survival show no benefit to the young from these associations and measurements of adult survival suggest likewise no benefit to either donor or recipient parents. Patterson suggests therefore that brood mixing is not adaptive and occurs when shelducks breed at unusually high densities. Fervent adaptationists will probably want more data before they are convinced, but critics of the so-called adaptationist programme will perhaps be relieved to find that animals sometimes simply make mistakes!

One of the main messages which comes out of this excellent book, and from other long-term studies like it, is that there are not only marked differences between populations but also between years within the same population. Such variability makes field work all the more interesting, but it means that we need to be careful before we generalize from the findings of short-term research projects. ☐

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The planets exposed

Dennis L. Hartmann

The Cambridge Photographic Atlas of the Planets. By Geoffrey Briggs and Frederick W. Taylor. Pp.255. ISBN 0-521-23976-1. (Cambridge University Press: 1982.) £12.50, \$24.95.

WE ARE nearing the end of a remarkable period of exploration of the Solar System. Six of the nine planets and their satellites have been the objects of close scrutiny by unmanned probes; pictures and data have been returned to Earth from the surfaces of our nearest planetary neighbours; men have walked on the Moon. All of this has taken place in the past 20 years, most of it in the past decade.

The early symbol of a new public awareness of the Solar System and our place in it was perhaps the sight of Earth from the vicinity of the Moon obtained by the Apollo astronauts. In *The Cambridge Photographic Atlas of the Planets* this perspective on our familiar planet and its satellite is supplemented by a large number of close-up photographs and other images of five additional planets and most of their satellites. It is this new view of the Solar System, obtained mostly from unmanned spacecraft, which Briggs and Taylor present in their book.

The authors begin with an overview of current ideas regarding the origin and subsequent evolution of the Solar System, directing particular attention towards the formation of the planets and the processes acting to determine the properties of their surfaces and atmospheres. This account provides the background for the six main chapters in which the planets are discussed in order of increasing distance from the Sun, beginning with the rather Moon-like planet Mercury and concluding with the intricate and still somewhat mysterious Saturnian System.

Each chapter opens with a section of text, first describing the overall characteristics of the planet and then delving into the scientific questions they present. Observations obtained by remote probes have provided many clues about the history and current state of the interiors, surfaces and atmospheres of the planets and their satellites, and these new findings are outlined both readably and authoritatively. Following the text comes a section of photographs and maps showing the surface or atmosphere of the planet in question and those of its satellites. The pictures, about half of them reproduced in colour, include some of the most spectacular and beautiful available, but many have also been chosen because they illustrate some interesting feature or provide some important clue about the body in question. A careful reading of the text heightens one's interest in the pictures, but lucid yet detailed captions mean that it is not necessary to read the text to appreciate

the significance of the illustrations.

The fascinating observations of the planets and their satellites illustrated and discussed in this book are likely to provoke the interest even of those with no previous exposure to planetary science. One can ponder such questions as whether there are continents on Venus and how the peculiar patterns in the clouds of its atmosphere are maintained. The surface features of Mars, the circulation of the atmosphere of Jupiter and the rings of Saturn provide equally challenging opportunities for inquiry and debate. Some readers, however, will be disappointed that no bibliography or source list is included to enable them to probe such issues more deeply.

Geoffrey Briggs and Frederick Taylor have a long-standing involvement in planetary exploration and write clearly and engagingly about their subject. Their book is likely to please a wide range of readers. The text should be comprehensible to the general reader, though it will be better understood and appreciated by those with some knowledge of the physical sciences. Because of its remarkable combination of strong visual appeal and scientific interest, *The Cambridge Photographic Atlas of the Planets* will be equally at home on the coffee table and in the research library.

Dennis L. Hartmann is an Assistant Professor of Atmospheric Sciences at the University of Washington, Seattle.

The noise of music

H.C. Longuet-Higgins

Music, Mind, and Brain: The Neuropsychology of Music. Edited by Manfred Clynes. Pp.430. ISBN 0-306-40908-9. (Plenum: 1982.) \$39.50, £24.89.

THE literature of music psychology — and *Music, Mind, and Brain* is no exception — deals with a wide variety of topics ranging from the neurophysiology of pitch perception to the affective responses of listeners. In order to bring some semblance of order to this vast area of inquiry it would therefore seem essential for anyone writing about the psychology of music to distinguish clearly at the outset between auditory stimuli in general and music in particular. The most interesting questions in the subject surely have to do with the differences between our responses to different pieces of music, but without a clear definition of what counts as a piece of music it is impossible to formulate such questions precisely. If music were simply — as Charles Lamb once complained — “the noise that they make on purpose”, then folk tunes, electronic squawks, animal noises and so on would have to be treated on an equal footing by the psychologist of music.

It seems to me that what distinguishes music from non-music is how we conceptualize it: a piece of music is a composition of notes, linked together by rhythmic and tonal relations, just as a sentence is a linguistic structure composed of words which are connected by syntactic and semantic relations. Modern theoretical linguistics became a science when this perspective finally came into focus, and thanks to the work of Chomsky and his successors it is now possible to see that the central problems of language have to do with the systematic relations between the detailed structures of utterances and the messages which they serve to convey. No psycholinguist would now dare to publish a paper which treated utterances or texts as unstructured sequences of noises or marks, and one might have hoped that an equivalent distinction would be taken for granted by psychologists of music. But with one or two laudable exceptions, the authors of *Music, Mind, and Brain* seem quite unaware of the need to explain, when they use the word “music”, exactly what they are talking about.

The book contains 21 chapters, together with a preface, a foreword and some appendices; a short sound sheet is tucked into the back of the book. The great majority of the contributions consist entirely of words, with no musical quotations; of the remainder only a handful make any attempt to describe in detail the rhythmic or tonal structures of the musical examples. In the first three chapters, for instance, Minsky, Pribram and Roederer discourse upon musical cognition, the

Wooden characters brought to book

Peter D. Moore

Identification of Modern and Tertiary Woods. By A.C. Barefoot and Frank W. Hankins. Pp.220. ISBN 0-19-854378-6. (Oxford University Press: 1982.) £47.50, \$98.

FROM the title of this book, one might be forgiven for thinking that the volume will provide a means for the identification of woods, both Tertiary and modern; in fact it does not. It sets out to illustrate those features of wood anatomy which can most usefully be applied to the characterization and identification of timber. It is, therefore, a guide for both students and timber technologists which will enable them to use with greater confidence some of the multiple-entry card keys currently on the market. Much of the book is actually based upon one of these keys, available on microfiche from Harvard University, which is itself a compilation of earlier keys and is still incomplete at present.

The opening chapter asks the question, “Why identify and study wood?”, but fails adequately to answer it. It is in any case a rather superfluous question since the reader presumably has his own reasons, but a survey of these reasons could be of general interest.

There follows an introductory chapter

on wood anatomy, preceding the more detailed analysis of key features used in wood identification. This account lacks structure in the way it deals with topics and fails adequately to define certain terms. Even such a straightforward expressions as “bark” are not clearly defined in the text and the drawings are sometimes so dense that the lines from labels are lost in them. More subtle terms, such as “roey-grained”, are used but not explained. One redeeming feature is the use of some excellent scanning electron micrographs, a technique which could have been further exploited and improved by detailed labelling — one photograph has letters attached but lacks an explanation in the legend. As an introduction to wood anatomy, one wonders whether this chapter is indeed aimed at the newcomer or, more likely, the experienced botanist.

The main body of the book explains each of the 86 wood characters used in the punch card key, sometimes a difficult task, especially when such characters as “late wood conspicuous” need to be interpreted. On the whole, this section of the book is very helpful, especially in its profuse use of high-quality micrographs and detailed line drawings; indeed, it is hard to imagine how the key could be tackled without its aid. Finally, the arrangement of the card key is explained and a timely caveat is given concerning the variability of wood within species as a consequence of both geographical range differences and variation in environmental factors.

Taken as an illustrated adjunct to the punch card key, this volume is an extremely valuable asset. It should not, however, be regarded as a textbook of wood anatomy or as an identification manual in its own right.

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● Elaine Morgan's *The Aquatic Ape: A Theory of Human Evolution* (reviewed in *Nature* 296, 511; 1982) is now available in the United States. The book is published by Stein & Day, and costs \$14.95.

● The second volume of *Cell Biology of Physarum and Didymium*, edited by Henry C. Aldrich and John W. Daniel, has recently been published by Academic Press. For a review of Vol. 1 (*Organisms, Nucleus, and Cell Cycle*) see *Nature* 298, 874; 1982. The second volume, entitled *Differentiation, Metabolism, and Methodology* costs \$52, £??.

mechanisms of auditory memory and other broad questions, but are lamentably vague about music itself. In two other chapters Clynes and his colleagues discuss the "essentic forms" of seven basic emotions, and the characteristic "pulse" by which a composer, they claim, hall-marks his compositions; but they fail to offer any empirical tests by which this claim could in principle be falsified.

Chapter 5, by Jackendoff and Lerdahl, is possibly the best in the book. These authors clearly recognize the central importance of structural concepts in the description of music, but even they omit to give any precise account of tonal relations, or to explain, for instance, the relation between the time signature of a classical composition and rhythmic structures of the individual bars. Of the remaining contributions, the most interesting are those of Diana Deutsch on the concept of Gestalt in music, of Sundberg on the expression of emotion in singing, and of Makeig on the theory of tonality. Makeig is familiar with the current theory of tonal relations but

seems to be unaware of the insights expressed by the late Deryck Cooke in his classic book *The Language of Music* (Oxford University Press, 1959).

Elsewhere, Balzano offers a group-theoretical account of "pitch sets", and rediscovers the three-dimensional tonal space first proposed by Longuet-Higgins in 1962, but does not illustrate his proposals with any interesting musical examples. And Terhardt, an acknowledged authority on auditory pitch perception, proposes a musically outrageous "root sequence" for the first few bars of *Tristan und Isolde*, in which the augmented 4th (F to B) in the famous chord is implicitly misrepresented as a diminished 5th (E sharp to B). In all it seems that some neuropsychologists of music have much to learn before their ideas need to be taken very seriously by competent musicians. □

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will settle the game, and that if this number of tosses is imagined to have been made, the resulting 2^{a+b-1} possible games (each equally probable) may be classified according to the winner in each case, the stakes then being divided accordingly. Thus the real game, of indeterminate length, is embedded in an imaginary game of fixed length. Apart from this novel idea, however, such a solution by enumeration is straightforward, but Pascal offered both an independent method based on expectations which is valid for any number of players and, in the *Traité du Triangle Arithmétique*, the solution for two players in terms of the binomial distribution. This, now the standard solution, though derived from the solution by enumeration, was proved by Pascal by induction applied to expectations. The binomial distribution was not given algebraically but by reference to the "arithmetical triangle" of binomial coefficients, whose properties Pascal elaborated in his *Traité* (whence the name Pascal's Triangle).

An original result of Pascal's, given in the *Traité*, is the combinatorial proof of the fundamental relation

$${}^{n+1}C_{r+1} = {}^nC_r + {}^nC_{r+1},$$

and this work also contains the first formal proof of the long-known binomial theorem for positive integral index.

Late in his *annus mirabilis* of 1654 Pascal suffered an intense religious episode which persuaded him to withdraw from society. Apart from some important work on the cycloid curve, he contributed nothing further of significance to mathematics. He died on 19th August 1662.

R.J. Nelson's book is an addition to the voluminous secondary literature on Pascal which will sit uneasily on a scientist's bookshelf. An examination of Pascal's psychology from the point of view of linguistic philosophy, it is itself written in a language that few will find easy. Thus:

To be sure, with its emphasis on the nonapodictic notion of figure and its composition in fragments, this most famous work of Pascal [the *Pensées*] does seem to lend itself to a theory of language skeptical of verbal mimesis, to a catachresis suggesting, at least to Marin, that Pascal views all language as aporetic, disjoined from the things, either objects or immutable ideas, that a mimological view of language posits as the referents or signifieds of words or signifiers.

It will be a while before the author's insights into the workings of Pascal's mind influence our understanding of Pascal's science, and that may be a pity, for Part I ("The Adversary") of his book is both interesting and accurate where it touches on scientific matters; but when the Two Cultures are divided by a common language it is inevitable. □

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Pascal's mind and Pascal's science

A.W.F. Edwards

Pascal: Adversary and Advocate. By Robert J. Nelson. Pp.286. ISBN 0-674-65615-6. (Harvard University Press: 1982.) \$22.50, £18.

BLAISE Pascal was born in 1623 in Clermont, France. In 1631 his father Etienne Pascal (himself an able mathematician who gave his name to the "limaçon of Pascal") moved to Paris in order to secure his son a better education. There, Etienne was one of the founders of Marin Mersenne's "Academy", the finest exchange of mathematical information in Europe at the time. To this informal academy he introduced his son at the age of 14, and Blaise immediately put his new source of knowledge to good use, producing (at the age of 16) his *Essay pour les Coniques* based on the work of Desargues, another founder-member of the Academy.

In the succeeding years the young Pascal designed and had built the first mechanical adding machine and conducted experiments into the nature of a vacuum, but his chief scientific contribution was to lay the foundations of the theory of probability.

The fame of Pascal, however, rests only to a small degree on his scientific work. Both adversary and advocate, as the title of this book suggests, he employed his exceptional talents in the public pursuit of religious controversy (especially through the *Lettres Provinciales*) and the compilation of extensive notes (the *Pensées*) intended as the basis of *An*

Apology for the Christian Religion which he did not live to complete. These works not only provide extraordinary insights into the human condition, but have had a great and lasting influence on French language and literature.

Yet his influence on probability was scarcely less; before his time, probability calculations amounted to no more than the enumeration of equally-probable outcomes in games of chance. Pascal introduced the important idea of manipulating expectations using recursively the fact that if expectations of gain X and Y are equally probable, the expectation is $\frac{1}{2}(X + Y)$. He also introduced the binomial distribution for equal chances and with its help, and that of mathematical induction applied to expectations, solved the Problem of Points for two players.

This problem was the topic of the correspondence between Pascal and Pierre de Fermat in the summer of 1654 which, together with Pascal's contemporary *Traité du Triangle Arithmétique*, includes not one but three solutions. Suppose two players stake equal money on being the first to win n points in a game in which the winner of each point is determined by the toss of a coin, heads for one player and tails for the other. If such a game is interrupted when one player still lacks a points and the other b , how should the stakes be divided between them?

On the evidence of the correspondence, Fermat and Pascal independently concluded that the problem could be solved by noting that at most $(a + b - 1)$ more tosses

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